# Commitment and Potential of Dental Pulp Stem Cells:

# Their Role in Directing Dental Tissue Regeneration

**A Thesis Completed in Fulfilment of the Requirements for the Degree of**

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***Every day, in every way, I'm getting better and better.***

***~ Emile Coue***

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*I would thank you from the bottom of my heart, but for you my heart has no bottom.*

*~ Author Unknown*

**Summary**

Dental pulp stem cells (DPSCs) play an integral role during dentine injury where they migrate towards the injury site, proliferate, differentiate into odontoblast-like cells and secrete a mineralised matrix, protecting the vital pulp tissue and preserving the tooth organ. Dentine matrix proteins (DMP) may have a role in stimulating this reparative dentine formation.

Multiple adult stem cells make up the DPSC population. Within this study progenitor cells were isolated from dental pulp by preferential fibronectin adherence (FNA) or using magnetic activated cell sorting (MACS) to isolate P75 expressing neural crest cells. Clonally expanded colonies were established. The proliferative FNA and P75-sorted populations were shown to be distinct progenitors with differing morphology and stem cell marker expression. One FNA clonal population was differentiated into mesenchymal lineages that established it as a multipotent DPSC population.

Clonal populations were supplemented with DMP *in vitro* to examine the potential role of DMP in modulating cell behaviour during dentine injury. Supplementing with DMP had a dose dependent response on DPSC viability, increased cell numbers, reduced apoptosis and promoted cell migration, suggesting that the growth factors in DMP may have a positive synergistic effect on DPSC behaviour.

Fluorescently stained DPSCs were microinjected into transverse tooth slices and placed in culture to develop an *ex vivo* model to study DPSC behaviour in a tooth environment. Injected cells remained viable after 7 days of culture, providing proof of concept data that DPSCs can be localised for study *in situ*.

This thesis was successful in the isolation of clonal populations representing progenitor cells with different characteristics. Supplementing DPSCs with DMP promoted cell behaviour facilitating reparative dentinogenesis, indicating a potential clinical application for DMP in restorative therapy. The study of DPSCs using the *ex vivo* model will be important in further development of these novel therapies for dental tissue regeneration.

## Abbreviations

| | |
|---|---|
| 2D/3D | 2/3 Dimensional |
| α-MEM | Alpha-Modification Minimal Essential Medium |
| ABC | Apical Bud Cell |
| AIM | Adipogenic Induction Medium |
| ALP | Alkaline Phosphatase |
| AMM | Adipogenic Maintenance Medium |
| ANOVA | Analysis of Variance |
| BMP | Bone Morphogenic Protein |
| BMSC | Bone Marrow Stromal/Stem Cell |
| bp | Base Pairs |
| BSP | Bone Sialoprotein |
| cDNA | Complimentary Deoxyribonucleic Acid |
| $Ca(OH)_2$ | Calcium Hydroxide |
| CFE | Colony Forming Efficiency |
| CFU-F | Colony Forming Unit-Fibroblasts |
| $CO_2$ | Carbon Dioxide |
| Col2a | Type II Collagen |
| CS/C4S | Chondroitin(-4-)Sulfate |
| dd.$H_2O$ | Double Distilled Water |
| DEPC-$H_2O$ | Diethylpyrocarbonate-Treated Water |
| DMP | Dentine Matrix Protein (fraction of the dentine matrix) |
| Dmp-1 | Dentine Matrix Protein-1 (a NCP of the dentine matrix) |
| DMP-Neg | Dentine Matrix Protein-Negative Control |
| DMSO | Dimethyl Sulfoxide |

| | |
|---|---|
| DNA | Deoxyribonucleic Acid |
| DPSC | Dental Pulp Stem Cell |
| Dpp | Decapentaplegic |
| DPP | Dentine Phosphophoryn |
| DSP | Dentine Sialoprotein |
| DSPP | Dentine Sialophosphoprotein |
| ECM | Extracellular Matrix |
| EDTA | Ethylenediaminetetraacetate |
| Eph | Ephrin (Signalling Pathway) |
| ESC | Embryonic Stem Cell |
| ET3 | Endothelin-3 |
| FACS | Fluorescence Activated Cell Sorting |
| FcR | Fragment Crystallisable Receptor |
| FGF | Fibroblast Growth Factor |
| FITC | Fluorescein Isothiocyanate |
| FNA | Fibronectin Adherent |
| GAG | Glycosaminoglycans |
| GSC | Germ-Line Stem Cells |
| GuCl | Guanidine Chloride |
| hFNA | Human Fibronectin Adherent |
| hi-FCS | Heat Inactivated Foetal Calf Serum |
| hNA | Human Non-Adherent |
| hP75 | Human LANGFR/P75 |
| HSC | Haematopoietic Stem Cell |
| IBMX | 3-Isobutyl-1-Methyl-Xanthine |

| | |
|---|---|
| ICC | Immunocytochemistry |
| IGF-1 | Insulin-Like Growth Factor I |
| IHC | Immunohistochemistry |
| IL | Interleukin |
| IMPDH | Inosine-5'-Monophosphate Dehydrogenase |
| IMS | Industrial Methylated Spirits |
| iPS | Induced Pluripotent Stem Cell |
| Klf4 | Krüppel-Like Factor 4 |
| KOH | Potassium Hydoxide |
| KS | Keratin Sulphate |
| LANGFR/p75 | Low-Affinity Nerve Growth Factor Receptor |
| LIF | Leukaemia Inhibitory Factor |
| LPL | Lipoprotein Lipase |
| LPS | Lipopolysaccharide |
| MACS | Magnetic Activated Cell Sorting |
| M-MLV | Moloney-Murine Leukaemia Virus |
| MMP | Matrix Metalloproteinase |
| MSC | Mesenchymal Stem/Stromal Cell |
| Msx1 | Msh Homeobox-1 |
| NA | Non-Adherent |
| NaOH | Sodium Hydroxide |
| NCP | Non-Collagenous Protein |
| NHS | National Health Service |
| OCN | Osteocalcin |
| Oct4 | Octamer-Binding Transcription Factor 4 |

| | |
|---|---|
| ON | Osteonectin |
| OPN | Osteopontin |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PD | Population Doubling |
| PDGF | Platelet Derived Growth Factor |
| PDL | Periodontal Ligament |
| PDLSC | Periodontal Ligament Stem Cell |
| PFA | Paraformaldehyde |
| PG | Proteoglycan |
| PPARγ2/PPARG2 | Peroxisome Proliferator-Activated Receptor Gamma isoform 2 |
| QD | Quantum Dots |
| rFNA | Rodent Fibronectin Adherent |
| RGD | Arg-Gly-Asp (Tripeptide Protein Motif) |
| rNA | Rodent Non-Adheret |
| RNA | Ribonucleic Acid |
| rP75 | Rodent LANGFR/P75 |
| RT-PCR | Reverse Transcription-Polymerase Chain Reaction |
| SCAP | Stem Cell of the Apical Papillae |
| SCID | Severe Combine Immunodeficiency |
| SEM | Standard Error of Mean |
| SGF (IGF-2) | Skeletal Growth Factor |
| SHED | Stem Cells from the Human Exfoliated Deciduous Teeth |
| Sox2/9 | SRY (Sex Determining Region-Y)-Box 2/9 |
| SSEA | Stage-Specific Embryonic Antigen |

| | |
|---|---|
| TBS | Tris-Buffered Saline |
| TGF-β | Transforming Growth Factor-Beta |
| UK | United Kingdom |
| USA | United States of America |
| UV | Ultra Violet |
| VCAM-1 | Vascular Cell Adhesion Molecule-1 |
| VEGF | Vascular Endothelial Growth Factor |
| Wnt | Wingless in Drosophila |

**Units of Measurement**

| | |
|---|---|
| % | Percentage |
| ℃ | Degrees Celsius |
| ×g | Gravitational Acceleration |
| U | Unit |
| N | Normal |
| M | Molar |
| mM | Millimolar |
| µM | Micromolar |
| nM | Nanomolar |
| g | Gram |
| mg | Milligram |
| µg | Microgram |
| ng | Nanogram |

| | |
|---|---|
| L | Litre |
| mL | Millilitre |
| µL | Microlitre |
| cm | Centimetre |
| mm | Millimetre |
| µm | Micrometre |
| nm | Nanometre |
| hr | Hour |
| min | Minute |
| s | Second |
| V | Volts |
| RLU | Relative Luminescence Unit |

**Contents**

# Chapter 1:

# Main Introduction

## 1.1. Introduction

Dental caries is a worldwide problem affecting approximately 80% of individuals at some point in their lifetime (Chadwick et al. 1999). This is despite the introduction of fluoride-based dentifrices and better oral healthcare in the Western world, with reports of caries reduction over the past 25 years (Downer 1993; Eriksen 1998). With a recent adult dental health survey in 2009, it reports a reduction in dental restorative treatment compared with the survey carried forth during the 1960s for England, Wales and Ireland. This has social and economic consequences for the affected countries with 20 million restorations placed every year, it costs the National Health Service (NHS) £173 million per annum in the United Kingdom (UK) alone (Murray 2011; NHS Centre for Reviews and Dissemination 1999) Treatment involves replacement of the diseased areas of the tooth with an artificial restoration. There are many types of restorations used clinically; dental amalgams were commonly used before the introduction of newer restorations, such as composite resins, glass ionomer cements and their variations. These more recent restorations have added benefits of being tooth coloured and are as resilient as traditional amalgam restorations. Other less commonly used restorations include porcelain, cast gold and other alloys (NHS Centre for Reviews and Dissemination 1999).

Despite an extensive list of dental restorations, all following a similar “drill and fill” procedure, unfortunately none of these artificial restorations provide secure

protection. At best, 58% of restorations survive after 10 years of placement (in a 2009 survey), resulting in over 60% of clinical restorative dentistry treatments being for the replacement of failed restorations (Burke and Lucarotti 2009; NHS Centre for Reviews and Dissemination 1999). The restorations are affected by the environment of the oral cavity and the lifestyle/characteristics of the patient. Failure in the restoration occurs when there are microleakages and openings in the restoration or at its perimeter. This can cause pulpal irritation from the movement of the filling, increased sensitivity from the irritation and fluid contact, and the influx of bacterial pathogens. The latter ultimately results in secondary caries, pulpal infections and if left undetected, can lead to systemic infections (Gallato et al. 2005; Goldberg et al. 2009; NHS Centre for Reviews and Dissemination 1999; Pashley 1996). Consequently the tooth would likely require endodontic root canal therapy. This process leaves the tooth brittle, susceptible to fractures and can ultimately lead to loss of the tooth. For this reason, alternative or more refined therapies are being sought after (Kahler et al. 2003; Kishen and Asundi 2002).

It is well established that the tooth itself has a capacity for natural dentine repair. Minor injury such as early caries can cause the pre-existing, post-mitotic, dentine secreting cells called odontoblasts, to upregulate dentine matrix production in a process known as reactionary dentinogenesis. This forms a protective barrier to the vital pulp tissue (Smith et al. 1995). With more severe cases of injury such as advanced caries, trauma and the placement of dental restorations, the necrosis of odontoblasts initiates a cascade of events to protect the pulp. It is well established that dental progenitors, the dental pulp stem cells (DPSCs), reside in the dental pulp (Shi et al. 2005; Sloan and Smith 2007). These cells migrate towards the injury site

and differentiate into odontoblast-like cells to replace the lost odontoblasts. These cells then secrete a protective matrix in a process known as reparative dentinogenesis; a repair process similar to the natural development of the dentine layer (Avery and Chiego 2006; Nanci 2003; Shi et al. 2005; Sloan and Smith 2007).

The odontoblast-like cells differentiate from DPSCs where clonogenic studies show variability in growth rates and differentiation ability of DPSCs to form dentine (Gronthos et al. 2002; Gronthos et al. 2000). A variety of techniques have been used for the isolation of mesenchymal stem cells (MSCs) such as the selection for surface antigen Stro-1 and CD146 markers (Shi et al. 2005; Sloan and Smith 2007), and neural crest stem cells have been selected by their low affinity nerve growth factor receptor (LANGFR) surface antigen (Waddington et al. 2009). CD146 is a cell adhesion antigenic marker for promoting vascularisation. The study of a negatively selected side population of these cells ($CD146^{-}$) harvested from the dental pulp were shown to be capable of promoting angiogenesis post-transplantation into a host site (Iohara et al. 2009; Iohara et al. 2006). This indicates other progenitors may be capable of revascularisation in the dental pulp, not just the $CD146^{+}$ cells. Collectively these studies illustrate there is more than one progenitor population in the dental pulp with varying differentiation potentials. Based on their differential and regenerative potential (Waddington et al. 2009; Yang et al. 2007a), it may be possible for these cells to be utilised in developing therapy for dentine repair, although the definitive cells required for this process remain unknown.

Central to the control of these DPSCs and to the regenerative process are cytokines and growth factors. These bioactive signalling proteins promote cell differentiation,

maturation and many other processes. There is an extensive list of growth factors proposed to partake in the dentine repair process. These include the transforming growth factor-β (TGF-β) super-family composed of various TGF-β isoforms and bone morphogenic proteins (BMPs), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). Each growth factor has demonstrated either improvement to or inducement of tissue repair (Atluri and Woo 2008; Baker et al. 2009; Begue-Kirn et al. 1992; Finkelman et al. 1990). Studies using whole dentine matrix protein (DMP) fractions have demonstrated its role in dentine repair, where the dentine matrix induces a reactionary and reparative dentinogenesis-like response (Smith et al. 1995; Smith et al. 1994). The use of a single growth factor may not be an accurate depiction of the complex interaction between the myriad of proteins in the dentine. A combination of growth factors may be a better representation of natural dentine repair, which may be used in developing novel therapeutic strategies.

A principle aim of this thesis is to investigate whether more than one population of progenitors reside in the dental pulp and if there are different characteristics between different populations. The cells will be isolated by two different methods and characterised for comparison. Isolation methods include fibronectin preferential adherence and magnetic activated cell sorting (MACS) to select for primitive cells and neural crest cells respectively (Jones and Watt 1993; Waddington et al. 2009). The proliferative and differential abilities of these cells will be explored to determine their clinical therapeutic value or for tissue engineering purposes. The thesis will then explore the effects of DMP on the DPSCs, if it causes any changes to the progenitor cell behaviour in terms of migration, viability and proliferation. The

results from such findings will indicate if the dentine matrix can be used to develop novel bioactive dentine repair therapies, accelerating/stimulating dentine regeneration and maintaining vitality of the dental pulp. Lastly, as an exploratory chapter, this thesis will develop a DPSC localisation method when the cells are implanted in an *ex vivo* tooth slice culture system (Hasegawa 1989; Sloan et al. 1998) which enables us to study DPSC behaviour in its tooth environment.

## 1.2. Architecture of the Tooth Organ

Teeth are the hardest structures of the mammalian body, making up approximately 20% of the oral cavity. The tooth structure is separated by a crown and root region, distinguished respectively as the visible teeth and non-visible areas below the oral mucosa (soft gum tissue). The crown of the tooth is covered by a heavily mineralised enamel layer (composed of 96-98% inorganic tissue) and the root is covered in a mineralised tissue sheath known as the cementum. The main body of the tooth is the dentine layer, located below the enamel and cementum layers, with vital pulpal chamber at its core (figure 1.1) (Avery and Chiego 2006; Nanci 2003).

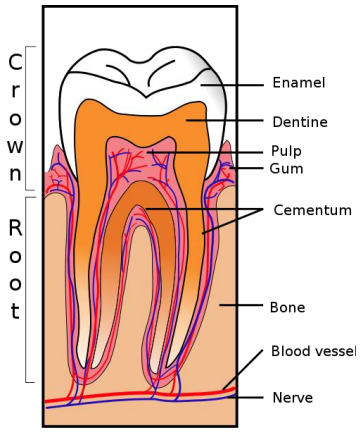



**Figure 1.1: Schematic of a tooth section with distinct enamel, dentine, cementum and soft pulp tissue layers (Image by Schmitt H.W., Wikimedia Commons: http://commons.wikimedia.org/wiki/File:Tooth_Section.svg).**

### 1.3. Development of the Tooth Organ

The embryonic-like neural crest cells play a crucial role in tooth development. These cells derive from the ectodermal primitive germ layer, forming at the cephalic (head) region of the neural plate during early embryogenesis. These cells then migrate from the dorsal side through a neural tube (precursor of the brain and spine) to reach other parts of the embryo where they then differentiate forming the five pairs of branchial arches. The first and second branchial arch pairs (figure 1.2) form head and neck structures by differentiating into (ecto)mesenchymal cells and develops into the salivary glands, bone, cartilage, nerves and musculature of the craniofacial organ. The first branchial arch is associated with forming the maxillary and mandibular appendages and progenitors of tooth mesenchymal cells (Avery and Chiego 2006; Bronner-Fraser 1993; Chai et al. 2000).

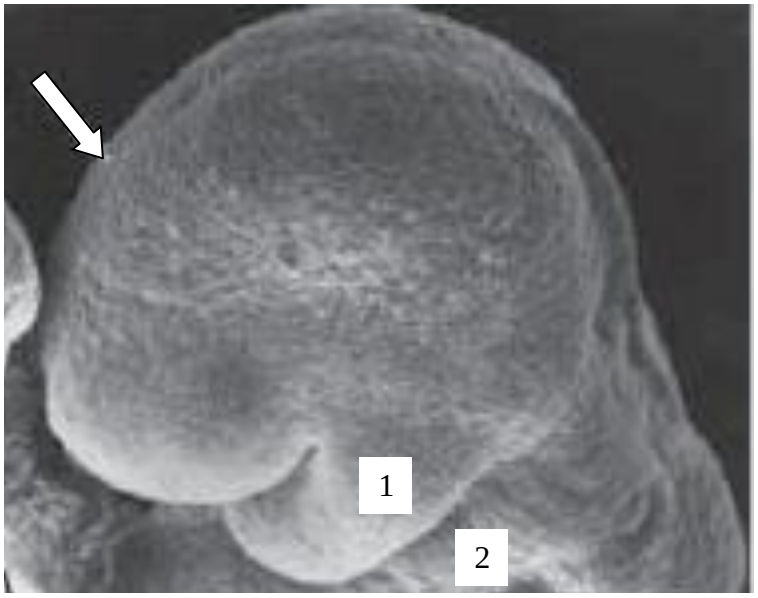



**Figure 1.2: Scanning electron microscope image of a mouse embryo where the neural plate has folded into the neural tube with a closed opening (anterior pore, shown with arrow) and the developing first (1) and second (2) branchial arches (Chai et al. 2000).**

The molecular and cellular control of tooth formation has not been fully established since suitable genetic markers are not yet recognised (Chai et al. 2000). Current work on the embryonic developmental *wnt1* gene shows promise as a neural crest genetic marker for tooth development as expressed by the neural crest cell population and as it migrates away from the neural tube differentiating into ectomesenchymal cells for tooth formation (Chai et al. 2000; Echelard et al. 1994; Soriano 1999).

Tooth formation initiates from the interaction between oral epithelial cells (dental lamina) and the cranial ectomesenchymal cells. As a tooth germ matures, it progresses through bud, cap and bell growth stages, with condensation of the mesenchyme to form the dental papilla (base of tooth) and consequently the dental pulp at the cap stage (Berkovitz et al. 2011). The formation of differentiated enamel-secreting ameloblasts (amelogenesis) and dentine-secreting odontoblasts (dentinogenesis) occurs at the bell stage. Crown growth and its mineralisation is followed by root growth and its mineralisation, where supporting tissues (the cementum, periodontal ligament and the alveolar bone) develop as the crown erupts into the oral cavity (summarised in figure 1.3) (Avery and Chiego 2006; Driessens and Woltgens 1986; Jernvall and Thesleff 2000).

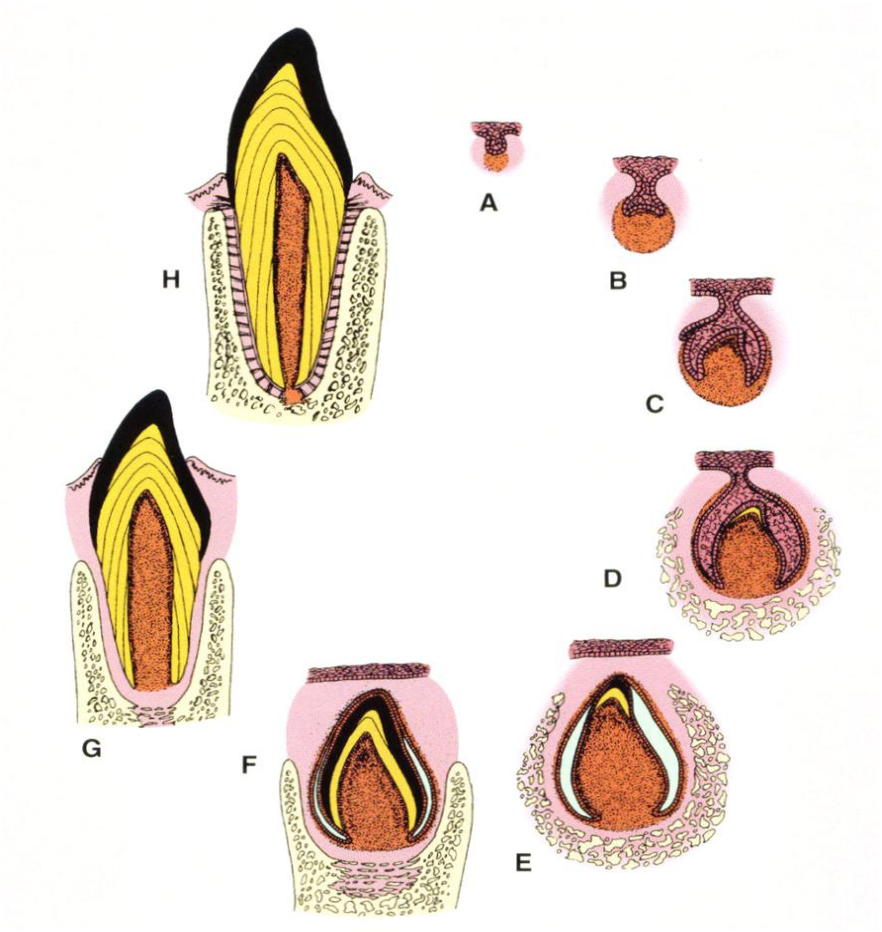



**Figure 1.3: A summary of tooth development through the bud (A), cap (B) and bell (C) stages, followed by dentinogenesis (D, yellow regions) and amelogenesis (E, black regions) until completion of the crown (F). The root develops as the crown erupts (G), continuing its growth and anchoring itself with periodontal ligament for complete formation of the adult tooth (H) (Avery and Chiego 2006).**

## 1.4. Components of the Dental Pulp

The pulpal tissue at the centre of the tooth can be divided into four distinct regions, a post-mitotic dentine-secreting odontoblast layer which surrounds the pulp, the cell-free zone of Weil which is an acellular region beneath the odontoblasts, separating it from a high density of cells (mainly fibroblastic cells) known as the cell-rich zone, and at the centre is the pulp core which houses the major blood vessels and nerves

(figure 1.4) (Arana-Chavez and Massa 2004; Driessens and Woltgens 1986; Fitzgerald et al. 1990; Iohara et al. 2009; Kitamura et al. 1992; Linde and Goldberg 1993). As nutrient transportation is vital for tissue function, blood capillaries are found in the sub-odontoblast layer, forming in between the odontoblasts and exchanging nutrients during early dentine formation (dentinogenesis). When dentine formation is complete, the vessels migrate out of the odontoblast layer, no longer supplying as large amount of nutrients. This supports previous reports that odontogenic precursors may originate from the perivascular tissues (Nanci 2003; Shi and Gronthos 2003).

Cells residing in the pulp include undifferentiated ectomesenchymal cells, fibroblasts, immune and nerve cells. The ectomesenchymal cells, or mesenchymal stem cells (MSCs), are found at the cell-rich area and pulp core and are typically polyhedral in morphology with a large nucleus. MSCs are normally closely associated with blood vessels (perhaps to be near the source of signalling molecules), and reduces in cell numbers with aging correlating with the reduction in regenerative potential of the pulp (Avery and Chiego 2006; Pinzon et al. 1966). Dendritic cells are present under the odontoblast layer (or around the odontoblasts in an unerupted tooth), making up about 8% of the pulp cell population, which is known to increase its numbers during dental caries to identify foreign bodies for immune cells (Keller et al. 2010; Mutoh et al. 2009; Nanci 2003). The immune cells of the pulp include macrophages, neutrophils, T-lymphocytes and B-lymphocytes, and are vital for the natural turnover of the pulp, as well as being part of the host defences during dental caries (Hahn et al. 1989; Izumi et al. 1995). These immune cells are known to be upregulated by immunocompetent odontoblasts with stimulation from bacterial

proteins such as lipopolysaccharides (LPS), flagellin and lipoproteins during pathogenic infiltration, and by pro-inflammatory cytokines naturally present in dentine such as interleukins (IL) and dentin sialoprotein (DSP), which are released during solubilisation of the dentine matrix by bacterial acids (Creagh and O'Neill 2006; Horst et al. 2009; Levin et al. 1999; Silva et al. 2004; Veerayutthwilai et al. 2007).

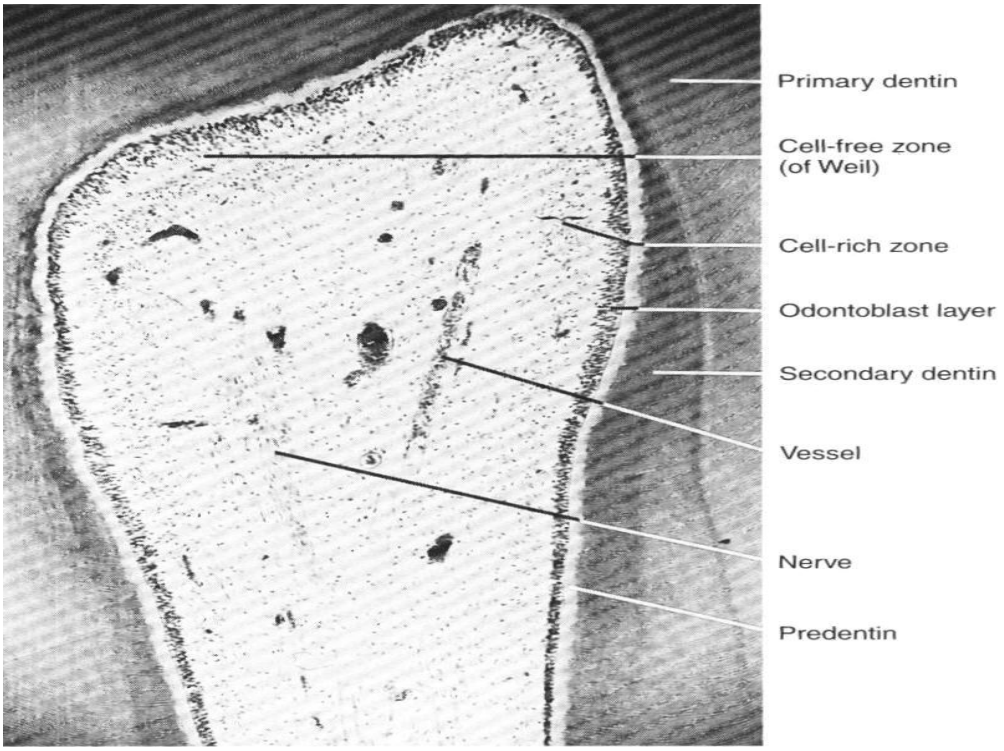



**Figure 1.4: Light microscope sections of a dental pulp and its distinct cell regions, nerves and vasculature (Nanci 2003).**

### 1.5. The Dentine Matrix

The dentine layer of the tooth is the calcified product of organic constituents secreted transiently or continuously by odontoblast cells, which do not undergo remodelling or resorption when compared with bone development. Dentine is composed of

approximately 30% organic and 50% mineralised tissue (the remainder is water), although these are not heterogeneously distributed throughout the dentine (Linde and Goldberg 1993). The dentine matrix contains densely arranged tubules, structures that contain elongated extensions of the odontoblast process (Tomes' fibre) where most secretion and absorption takes place. This provides vitality to the dentine, but also makes the tissue extremely permeable to fluids, external stimuli and susceptible to microbial invasions (Avery and Chiego 2006; Linde and Goldberg 1993).

Dentine extracellular matrix (ECM) consists of hydroxyapatite plates with mainly collagen and an extensive list of known non-collagenous proteins (NCPs) which affect dental stem cell fate but also maintains its surrounding cells. Isolated odontoblasts supplemented with DMP retain their morphology for up to four days in culture, whereas without DMP supplementation the cells acquire a fibroblastic morphology, and DMP is suggested to have a chemotactic response to the odontogenic precursors during dentine repair (Lesot et al. 1986; Smith et al. 1995; Smith et al. 1994).

The dentine layer is made up of mainly type I and type V collagen fibres (Bronckers et al. 1986; Niyibizi and Eyre 1994). These collagen fibres enclose matrix proteins such as osteonectin (ON), which is found in odontoblasts and is thought to be involved with linking matrix and mineral during tissue mineralisation. ON is also upregulated during dentine repair where it may have an inhibitory effect during tertiary dentine formation (Bègue-Kirn et al. 1994; Itota et al. 2001; Ruch et al. 1995). Dentine phosphoprotein (DPP) is a chemoattractant to DPSCs (Yasuda et al. 2008), and along with bone sialoprotein (BSP), both have roles in initiating

mineralisation (Bianco et al. 1993; Boskey et al. 1990; Butler and Ritchie 1995; Linde and Goldberg 1993), where the mineralised tissue formation is regulated by dentine matrix protein-1 (Dmp-1 or AG-1) and DSP (Bronckers et al. 1994; Butler and Ritchie 1995; D'Souza et al. 1992; George et al. 1994), and remodelled/regulated by osteopontin (OPN) and osteocalcin (OCN), where both may act as chemoattractants to osteoclasts or aid its attachment (Butler 1989; Denhardt and Guo 1993; Gorter de Vries et al. 1987; Lian et al. 1986).

Aside from the matrix proteins, growth factors entrapped within the dentine matrix include fibroblast growth factor (FGF), where the FGF-2 isoform is suggested to increase proliferation and DSP formation with DPSCs (Nakao et al. 2004). Platelet-derived growth factor (PDGF), insulin-like growth factor I (IGF-1) and skeletal growth factor (SGF or IGF-2) are suggested to influence tooth regeneration and regulation, with roles in chemotaxis and odontoblast differentiation (Finkelman et al. 1990; Lovschall et al. 2001; Tanikawa and Bawden 1999; Wang et al. 1994; Yokose et al. 2004). The angiogenic factor, vascular endothelial growth factor (VEGF) is vital for pulp repair and enhanced dental healing (Grando Mattuella et al. 2007; Roberts-Clark and Smith 2000; Zhang et al. 2011), with a dose-dependant pro-angiogenic response when present at lower concentrations (Zhang et al. 2011).

The TGF-β family of growth factors have roles in tooth repair and development (Baker et al. 2009; Begue-Kirn et al. 1992; Nakashima et al. 1998; Sloan and Smith 1999). It is recognised that the TGF-β2 isoform plays a large role in tooth development, whilst TGF-β1 with BMP-2 have significant roles during the mineralisation process (Chai et al. 2003; Chai et al. 1994; Chai et al. 1999;

Nakashima et al. 1994; Ruch et al. 1995). The TGF-β family, particularly TGF-β1, have a role in odontoblast differentiation shown with *in vivo* and *in vitro* studies, as part of reparative dentinogenesis and dentine development (Baker et al. 2009; Cassidy et al. 1997; Graham et al. 2006; Smith et al. 1995)

### 1.5.1. Primary and Secondary Dentine Formation

During early tooth development, odontoblast cells elongate to produce a cytoplasmic process. This gradually "pushes" the odontoblasts from the dentine-enamel junction towards the pulp. Formation of the dentine matrix is established by early odontoblast secretion of fibronectin and type III collagen fibrils (von Korff's fibres) that are between 0.1 to 0.2 μm in diameter known as the mantle predentine (Avery and Chiego 2006; Pashley 1996; Tziafas et al. 2000; Yoshiba et al. 1994).

The predentine calcifies at a mineralisation front as the odontoblast matures and collagen type I is produced which acts as the scaffold for dentine development, with NCPs such as DPP and the dentine specific DSP (two most abundant NCPs in dentine), regulating dentine mineralisation (Godovikova and Ritchie 2007) and remodelled with proteins such as OCN and BSP (Butler and Ritchie 1995).

The small leucine-rich proteoglycans (PGs), decorin and biglycan as well as glycosaminoglycans (GAG) chains, particularly condroitin sulphate (CS) and keratin sulphate (KS) are thought to be involved with regulating mineralisation by its GAG chain affinity to TGF-β1. These are identified in pre-dentine and thought to be metabolised prior to conversion to dentine, perhaps by the release of growth factors (Baker et al. 2009; Butler and Ritchie 1995; Embery et al. 2001; Milan et al. 2004,

2005). However these proteoglycans are also contested to be involved with apatite crystal formation (Dechichi et al. 2007). Odontoblast secreted predentine and its mineralisation is shown in figure 1.5.

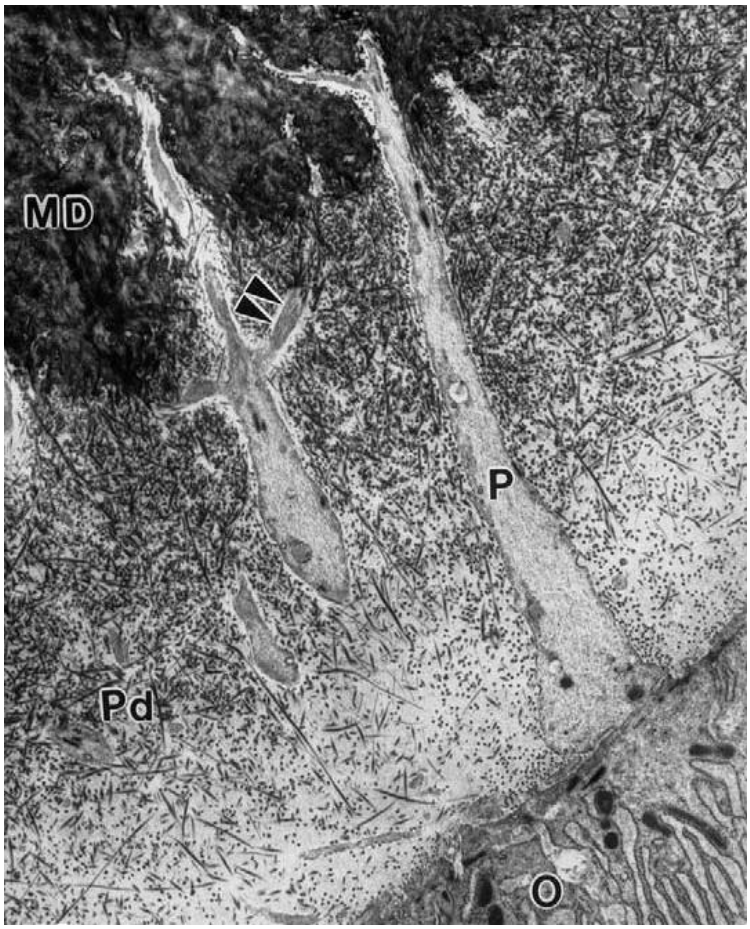



**Figure 1.5: Electron micrograph of odontoblast (O) secretion of collagenous non-mineralised predentine (Pd) through the odontoblast processes (P), which may have branching (double arrowheads). Areas of mineralised dentine (MD) shown by the darker image areas. Image ×5400 magnification (adapted from Arana-Chavez and Massa, 2004)**

The two major types of dentine secreted are primary dentine and secondary dentine. Primary dentine is secreted before the completion of root development, making up most of the mass of the tooth surrounding the pulp (Nanci 2003; Smith et al. 1995;

Smith and Lesot 2001). Secondary dentine has similar mineral and organic content and is secreted after the tooth root has developed, but at a much reduced rate which maintains dentine homeostasis. The secondary dentine is observable as it forms non-continuous extension of tubules to the primary dentine (figure 1.6) (Smith et al. 1995; Smith and Lesot 2001).

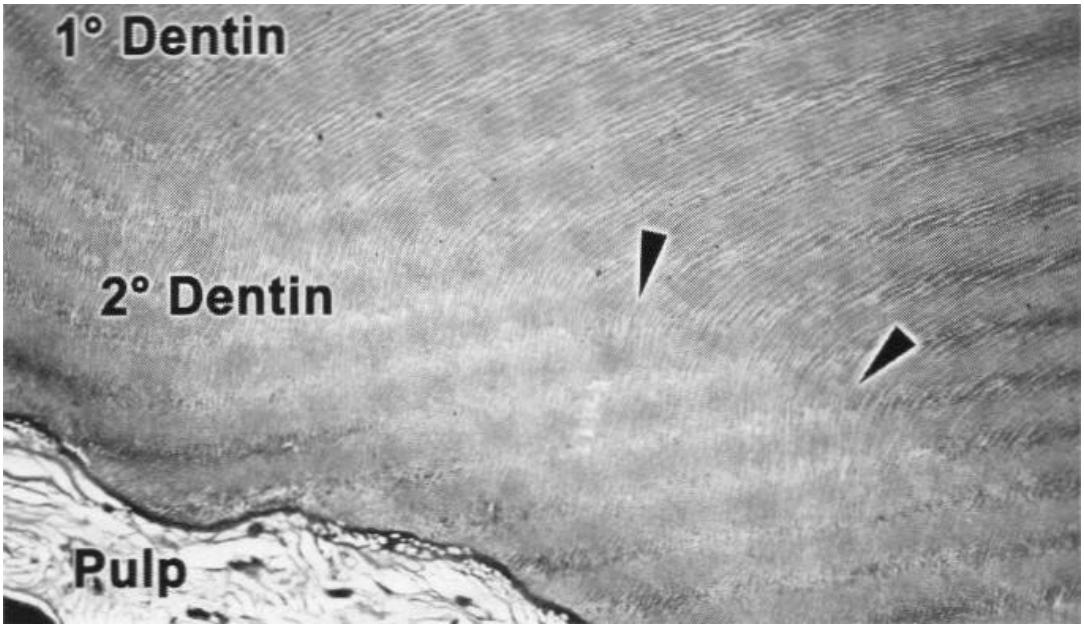



**Figure 1.6: Secretion of secondary dentine is non-continuous to the primary dentine. Arrowheads indicate the junction between primary and secondary dentine (Nanci 2003).**

### 1.5.2. Tertiary Dentine and Tooth Repair

Tertiary dentine is produced following dentine injury and is secreted at the injury foci. The secreted dentine can be tubular, following in parallel to the primary/secondary dentine, or it may also occur more sparsely (figure 1.7), irregularly or even absent altogether (Murray et al. 2000; Nanci 2003; Smith et al. 1995). Tertiary dentine has less collagen content, but with more NCPs such as BSP and OP (perhaps to aid rapid mineralisation) and forms a protective barrier at the

injury sites. There are two types of tertiary dentine, reactionary or reparative dentine, dependant on the severity of injury if the existing odontoblasts become damaged (figure 1.8).

Reactionary dentine is secreted from the viable, post-mitotic odontoblasts after mild injury, such as attrition (wear of teeth) and mild caries (decay). This is an autocrine process, believed to be initiated by BSP and other dentine matrix components and is upregulated by growth factors such as TGF-β and BMP (Begue-Kirn et al. 1992; Sloan and Smith 1999; Smith et al. 1995).

The growth factors are believed to be released from the ECM compartments by solubilisation with acids (via bacterial metabolism), which diffuse down dentine tubules and upregulate odontoblast activity (Smith et al. 1995; Tziafas et al. 2000). Studies have shown with the use of ethylenediaminetetraacetate (EDTA) solubilised bioactive molecules stimulate an increased deposition of reactionary dentine when applied onto tooth defects, when compared with treatment with other restorative materials (Duque et al. 2006).

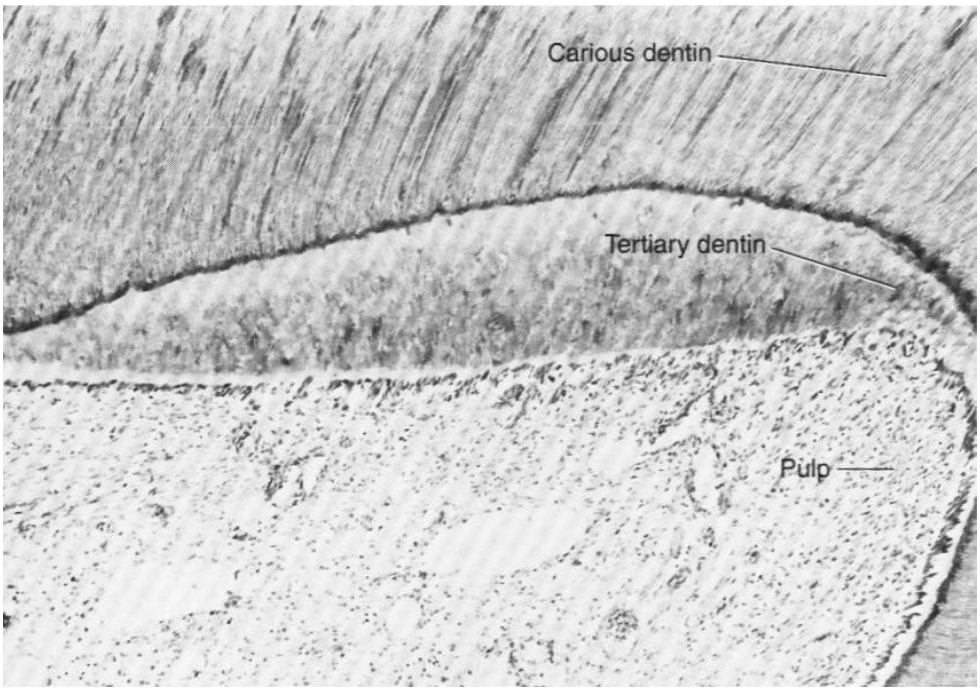



**Figure 1.7: Tertiary dentine secretion in response to caries, with sparse dentinal tubules (Nanci 2003).**

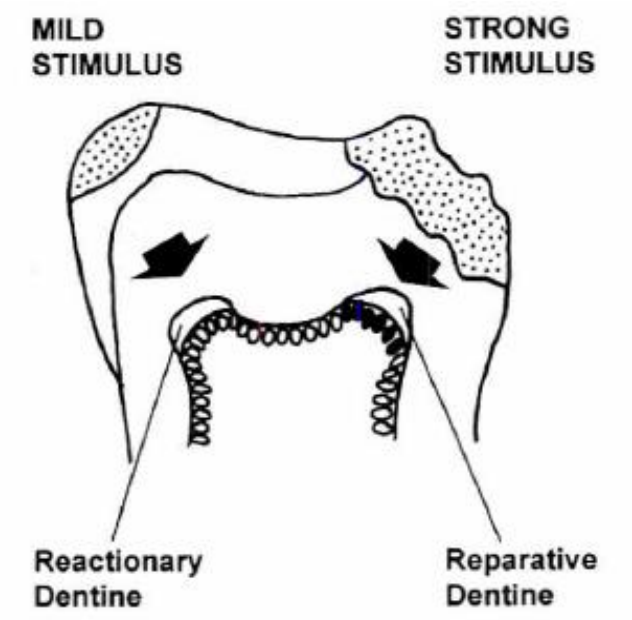



**Figure 1.8: Reactionary dentine is secreted by odontoblasts with mild injury, and reparative dentine is secreted from odontoblast-like cells after severe injury (Smith et al. 1995).**

Reparative dentinogenesis occurs after severe trauma to the odontoblasts, such as fractures and with placement of dental restorations. Death of the underlying odontoblasts causes a cascade of events to occur, involving the mitosis of precursor stem cells in the pulp and its migration to the subodontoblast layer with chemotactic stimuli (Tziafas et al. 2000). After the second mitosis of the precursor cells, one daughter cell differentiates into odontoblast-like cells for the synthesis and secretion of dentine matrix and the other daughter cell remains undifferentiated. These precursor cells (or preodontoblasts) are thought to be pericytes, MSCs, fibroblasts or maybe a unique dental pulp progenitor, although it remains undetermined as to which is the progenitor(s) necessary for successful tooth regeneration (Fitzgerald et al. 1990; Gronthos et al. 2000; Sloan and Smith 2007; Smith et al. 1995).

The differentiation of odontoblast precursors, the dental pulp stem cells (DPSCs), into odontoblast-like cells during reparative dentinogenesis is not fully understood. Unlike early odontoblast development, there is an absence of epithelial cells required for the epithelial-mesenchymal interaction that initiates odontoblast differentiation. It has been suggested that the epithelial cytokines necessary for differentiation may be entrapped within the dentine matrix during its formation and are released during injury, although it is uncertain how this is regulated (Nanci 2003; Sloan and Smith 2007; Smith and Lesot 2001). This is shown by the effect of adding dentine matrix components to dental derived progenitors, where DPSCs differentiate and secrete mineralised matrix, similar to the *in vivo* tertiary dentinogenesis process, and causes immature cells from the dental papillae to differentiate into odontoblast-like cells (Bègue-Kirn et al. 1994; Duque et al. 2006; Sloan and Smith 1999; Smith et al. 1994). Study of DPSCs and its manipulation by tissue engineering can lead to further

understanding of tooth development and repair, and lead to developing novel treatment modalities.

### 1.6. Stem Cells

Stem cells are the undifferentiated cell populations of the body which are able to proliferate with seemingly endless self-renewal and differentiate into terminally differentiated cells to maintain tissue homeostasis (Stocum 2001). Stem cells are excellent tools for tissue engineering. If stem cells can be harnessed, directed and regulated, they can be used for understanding progenitor behaviour that are naturally in the tooth and to develop novel regenerative therapy as part of tooth repair. Tissue engineering experiments have utilised embryonic stem cells (ESCs) and the more differentiated adult stem cells, where the latter is preferred as ESCs are more difficult to obtain due to ethical constraints (Stocum 2001).

#### 1.6.1. Embryonic Stem Cells

ESCs have a high self-renewal capacity and an ability to differentiate into all adult tissues including those of the germ line. ESCs originate from the pre-developed embryo and its differential potential makes them totipotent cells (Brook and Gardner 1997; Evans and Kaufman 1981; Martin 1981). These cells have the ability to differentiate into all of the body's cell types, including that of the extra-embryonic tissue, such as the placenta, vital for embryo development (Dickens and Cook 2007; Fuchs and Segre 2000; Mimeault and Batra 2006; Morrison et al. 1996; Thomson et al. 1998; Weissman 2000).

ESCs possess characteristically high telomerase enzyme expression levels, essential for extending the cell replicative life-span. The ends of a chromosome consist of tandem chromatin TTAGGG repeats known as telomeres, important for chromosomal stability and the overall cell vitality. With each cell division, there is a reduction in telomere repeats, due to incomplete chromosomal replication by the DNA polymerase. This results in shortening of the chromosomes, causing cell senescence and producing age-related disorders. Telomerase is an enzyme that adds telomere repeats to the ends of the chromosomes, enabling the cell to replicate indefinitely, compared with somatic cells which have low or no telomerase expression (Flores et al. 2006; Rudolph et al. 1999).

ESCs also express characteristic embryonic transcription factors, where four "factors" are deemed the most important: octamer-binding transcription factor 4 (Oct4), SRY (sex determining region Y)-box 2 (Sox2), Krüppel-like factor 4 (Klf4) and c-Myc. Oct4 which is part of the well-conserved eukaryotic homeodomain protein family, Sox2 maintains or preserves developmental potential, both are shown to activate transcription of fibroblast growth factor (FGF)-4, vital for extraembryonic (placental) tissue formation and embryo development. Oct4 or Sox2 knock-outs lead to a non-pluripotent and non-proliferative blastocyst during embryo development (Avilion et al. 2003; Nichols et al. 1998). Klf4 and c-Myc regulate gene expression, where upregulation of either Klf4 or c-Myc is shown to prevent differentiation and promote self-renewal of the ESCs (Cartwright et al. 2005; Li et al. 2005). These four factors are shown to be important with induced pluripotent stem cell (iPS) experiments, where transfection of all four factors into an unmodified fibroblast

"reprogrammed" the cell into a pluripotent state (Meissner et al. 2007; Takahashi et al. 2007; Takahashi and Yamanaka 2006).

Cells from a fully developed embryo blastoderm, blastocyst inner cell mass or primordial germ lines are pluripotent ESCs. These contain characteristic ESC markers such as stage-specific embryonic antigens (SSEA)-3 and -4, high telomerase enzyme activity and expression of transcription factors for Oct-3/4. The pluripotent ESCs are able to differentiate into a multitude of cells of the primitive endoderm, mesoderm and ectoderm germ cell layers of the body. However, these are unable to form a complete living organism on its own due to its inability to form extra-embryonic tissues like the totipotent cells. These cells are shown to differentiate randomly during *in vitro* culture, able to form teratomas when injected into severe combined immunodeficiency (SCID) mice where the inability to regulate itself produced structures from all three germ layers (Stocum 2001; Thomson et al. 1998).

However, *in vitro* supplementation with cytokines can maintain its stem cell potential. Leukaemia inhibitory factor (LIF), a pleiotrophic cytokine (influences many phenotypic effects) produced in many tissues (e.g. blastocyst, thymus and lungs), has a recognised role in maintaining murine ESC totipotency, including the upregulation of Klf4 (which upregulates Oct4), and c-Myc (Cartwright et al. 2005; Li et al. 2005; Metcalf 2003; Raz et al. 1999; Williams et al. 1988). The use of human embryos has ethical implications and has tumour-forming properties which makes alternative stem cells, such as adult stem cells, more desirable for tissue engineering purposes (Liao 2005).

### 1.6.2. Adult Stem Cells

Adult stem cells are multipotent progenitor cells from adult tissues, where isolation of these cells has less ethical restraints when patient consent is provided. Progenitor cells are defined as daughter cells derived from an asymmetric "mother" adult stem cell division. The "mother" stem cell has a high capacity for (infrequent) symmetric self renewal and is more rarely found within the tissues. Progenitors have a more limited ability to self renew making them transit amplifying cells, before external stimuli causes them to terminally differentiate into post-mitotic cells (figure 1.9) (Sloan and Waddington 2009; Stocum 2001). Some of the known adult stem cells include neural crest stem cells and the perivascular MSCs.

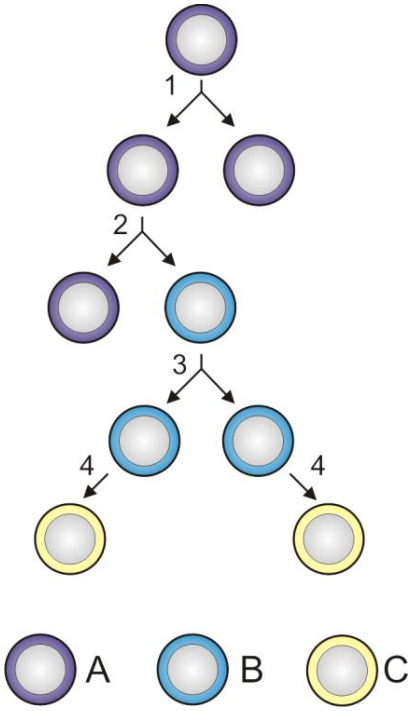



**Figure 1.9: Adult stem cell formation and differentiation. Where stem cells (A) normally replicate symmetrically (1), but stimuli causes asymmetric division (2) producing daughter progenitors (B). The progenitors also replicate (3) before forming terminally differentiated cells (C). (Image by Wykis from Wikimedia Commons: http://en.wikipedia.org/wiki/File:Stem_cell_division_and_differentiation.svg)**

#### 1.6.2.1. Neural Crest Stem Cells

During embryonic development, the neural crest is formed at the neural plate with activation of neural crest markers which include Slug, Snail and Twist, for neural crest proliferation, migration and initiation of the epithelial-mesenchymal interaction (Aybar et al. 2003). Neural crest cells can differentiate into non-ectomesenchymal tissues, such as neurons and pigment cells (Graham 2003), and also differentiate into ectomesenchymal cells, which are involved with tooth development and the eventual formation of odontoblasts (Chai et al. 2000).

It has been shown that neural crest stem cells are affected by environmental stimuli which affect the cell fate. However, the cues required for directing neural crest fate is not yet fully defined, where neural crest cell fate is naturally determined by influences of its environment as it migrates. During normal development, the early-migrating neural crest cells migrate to the dorsal (rear "back") and ventral (front "belly") ends of the branchial arches, whilst the late-migrating neural crest migrates only to the dorsal end. By swapping the two populations, manipulation of the early and late migrating neural crest cells show they are able to be substituted for each other as long as the environmental cues are present (Baker et al. 1997).

Culturing in mineralising conditioned media containing dexamethasone (essential, and it modulates BMP-2), ascorbic acid (cofactor required for hydroxylation and collagen formation), and β-glycerophosphate (source of inorganic phosphate for mineralisation), neural crest cells were shown to differentiate into skeletal cells (bone and cartilage cells) and grafting neural crest cells into developing chick-embryos with removed neural tubes, shows successful formation of cartilage tissue (Abzhanov

et al. 2003; Jäger et al. 2008; McGonnell and Graham 2002; Vater et al. 2011). This suggests a tissue engineering potential of directing these immature cells into required cell types with the correct environmental cues, such as into odontoblasts for development of dental repair. Previous work has already shown isolated neural crest stem cells are capable of differentiating into odontoblast-like cells *in vitro* (Chai et al. 2000; Dupin et al. 2007; Le Douarin and Dupin 2003; Peters and Balling 1999).

However, reports suggest neural crest cells are a heterogeneous population with differing proliferation and differentiation potentials with its cell-fate possibly restricted during early development (Dupin et al. 1998; Henion and Weston 1997). More alarmingly, neural crest cells that terminally differentiate into the pigment cell melanocyte, apparently de-differentiate and differentiate (or maybe trans-differentiate) into glial cells. Formation of neural crest melanocytes require endothelin-3 (ET3) signalling, where addition of ET3 to clonal cultures of melanocytes produced rapid cell division and a final culture of melanocytes and 40% glial colonies (Dupin et al. 2000), highlighting the instability of differentiated neural crest cell *in vitro*. Taking into account its heterogeneity and differentiation instability, the immature and embryonic-like population of neural crest cells are naturally involved with tooth formation, making them possible candidates for developing tooth repair therapies and for tissue engineering manipulation.

#### 1.6.2.2. Mesenchymal Stem Cells

One type of multipotent adult stem cell is the adherent and fibroblast-like MSC, which derives from the mesoderm and develops from the neural crest. MSCs are

proposed to have twice the amount of β1 integrins, cell surface receptors with a high affinity for the matrix protein fibronectin (Jones and Watt 1993) and can be isolated from connective tissues such as the knee synovial membrane, bone marrow, adipose and dental tissues (Barry and Murphy 2004; De Bari et al. 2001; Huang et al. 2009; Ikeda et al. 2010; Shirasawa et al. 2006). Isolated MSCs are able to form colonies as colony forming unit-fibroblasts (CFU-Fs) and differentiate into cells of other connective tissues, including adipocytes, chondrocytes and osteoblasts. As MSCs are found in a myriad of mesenchymal tissues, it is not yet fully recognised if they are a homogenous cell population, which may contain more mature forms of neural crest cells, and if MSCs from different tissues are the same type of cells (De Bari et al. 2001; Fuchs and Segre 2000; Wagers and Weissman 2004). MSCs are internationally defined as a plastic adherent cell, expressing certain surface antigens (some listed below) with an ability to differentiate *in vitro* into adipogenic, chondrogenic and osteogenic lineages (Dominici et al. 2006).

Of the most common surface antigens found, markers that are negatively expressed by MSCs include haematopoietic markers such as surface antigen CD34, immune cell surface protein CD14 and protein tyrosine phosphatase CD45 (Davies et al. 2010; Rastegar et al. 2010; Stocum 2001). However, several markers are unclear or appear to show different results depending if sourced from human or a rodent species, such as CD34 found in murine but not human isolated MSCs (Kolf et al. 2007). Positive MSC markers include the surface antigen Stro-1, conserved tyrosine kinase protein domains SH-2 and -3, conserved surface protein CD90 (Thy-1), and membrane glycoprotein CD105 (endoglin) (Davies et al. 2010; Jones et al. 2002; Rastegar et al. 2010; Yang et al. 2007b). MSCs also express embryonic markers such

as SSEA-4, Oct4 and Nanog (Gang et al. 2007; Kerkis et al. 2006), although expression varies with donor age, with increase in age causing decrease in multipotent differentiation potential (D'Ippolito et al. 1999). With such an extensive list of possible MSC markers, and as a single definitive marker for MSCs has not been recognised, multiple MSC markers is commonly used (Kolf et al. 2007). Knowledge of the positive markers for MSCs not only allows for its characterisation, but also for its isolation (such as antigen-based cell sorting methods) for tissue engineering purposes.

The MSCs are thought to express a variety of proteins and genes (or "factors"), where 98% of the MSC population is positive for the osteogenic *cbfa1* (*runx2*) transcription factor (Stocum 2001), suggesting a pre-programmed cell commitment. Extracellular signalling cytokines or growth factors that maintain the self-renewing and undifferentiated state of these adult stem cells include FGF, suspected to maintain cells in an undifferentiated state in similar pathways to maintaining undifferentiated limb buds during limb formation (Niswander 2002). Also Wnt proteins (particularly Wnt3a) are found to be vital during development, expressed at the inner cell mass of blastocysts (Barrow et al. 2007; Liu et al. 1999) which suppress differentiation of MSCs, maintaining their multipotency and increasing their proliferation (Boland et al. 2004) as well as the already mentioned LIF cytokine (section 1.6.1). MSCs also express certain embryonic genes, such as *oct-4*, *sox-2* and *rex-1*, which are just some of the important transcription factors found during embryonic development (Kerkis et al. 2006; Kolf et al. 2007; Pesce et al. 1998). These extracellular signalling molecules and transcription factors are recognised to maintain MSC multipotency. This is important for understanding stem cell potency

and for use in tissue engineering such as factors that maintain stem cell potency and for cell manipulation such as iPS technology.

### 1.7. Asymmetric Stem Cell Kinetics

As pluripotent stem cells divide, it gives rise to a clone and a multipotent or unipotent daughter cell, which may terminally differentiate into one of many or a single cell lineage respectively, to maintain tissue homeostasis (figure 1.9). This makes maintaining stem cell potential *in vitro* a difficult task, where dividing cells may naturally undergo asymmetric cell kinetics forming a population of variable cell commitment and also leading to eventual cell senescence.

All somatic cells have a finite ability for cell population doubling (PD) before reaching senescence, where the cell suffers from intracellular disorganisation and no longer synthesises new DNA. The cell remains in G1 phase of the cell replication, preventing proliferation which leads to eventual apoptosis (Stanulis-Praeger 1987). To utilise adult stem cells for tissue engineering, mechanisms that govern asymmetric cell kinetics need to be understood to allow their expansion in culture.

Two genes, p53 and inosine-5'-monophosphate dehydrogenase (IMPDH) are well known to have a role in governing asymmetric cell differentiation and regulating cell senescence. The p53 tumour suppressor gene is thought to be one genetic marker that directs asymmetric cell kinetics. Where p53-knockout mice yield symmetric cell division, the formation of two dividing daughter cells, this effectively abolishes natural senescence from occurring (Rambhatla et al. 2001; Sherley et al. 1995). IMPDH encodes for the rate-limiting enzyme for the synthesis of guanine

nucleotides, part of the ribonucleotide bases required for DNA/RNA synthesis. IMPDH is proven to be upregulated in tumourogenic cells (Fellenberg et al. 2010). IMPDH is also downstream of p53, as p53 expression causes downregulation of IMPDH which leads to asymmetric kinetics (Liu et al. 1998b) and forced IMPDH expression (by transfection with an IMPDH cDNA expression plasmid) negates p53 effects and leads back to symmetric cell kinetics (Liu et al. 1998a).

Three other proteins known to have an influence on asymmetric cell differentiation are the cyclin-dependent kinase inhibitor p21 and two other known tumour suppressors, p63 and Pten. However, the exact roles these proteins have on asymmetric kinetics remains less certain (Sherley 2002). Understanding the mechanisms for maintaining stem cell potency and maintaining a homogenous proliferative culture are important aspects for controlling stem cell behaviour. Prevention of senescence is important for maintaining a stem cell culture for use in tissue engineering studies.

Another aspect of the cell differentiation kinetics is its differentiation ability to form another cell lineage after it has already differentiated. Stem cells were classically thought to differentiate in a linear and committed route into terminally differentiated cells. Adult stem cells have been shown to be highly plastic, capable of forming into many cell lineages when influenced by its environment (Clarke and Frisén 2001). It is thought differentiated cells of one tissue type maintains the genes for different tissue types, which allows them to form into cells of a different tissue type by "de-differentiating" to an earlier ancestral cell line. This can then differentiate into another cell line, or perhaps by traversing across cell lineages by "trans-

differentiation" from cell type to another (example model see figure 1.10) (Blau et al. 2001; Sherley 2002). The neural crest derived melanocytes, are influenced by extracellular signalling that causes them to undergo trans-differentiation into glial cells, as mentioned previously (section 1.6.2.1). Producing a more desirable cell lineage from a less desirable lineage may be a valuable tool for tissue engineering and dental regenerative therapy, although the environmental signalling factors that are required to do this are not yet understood.

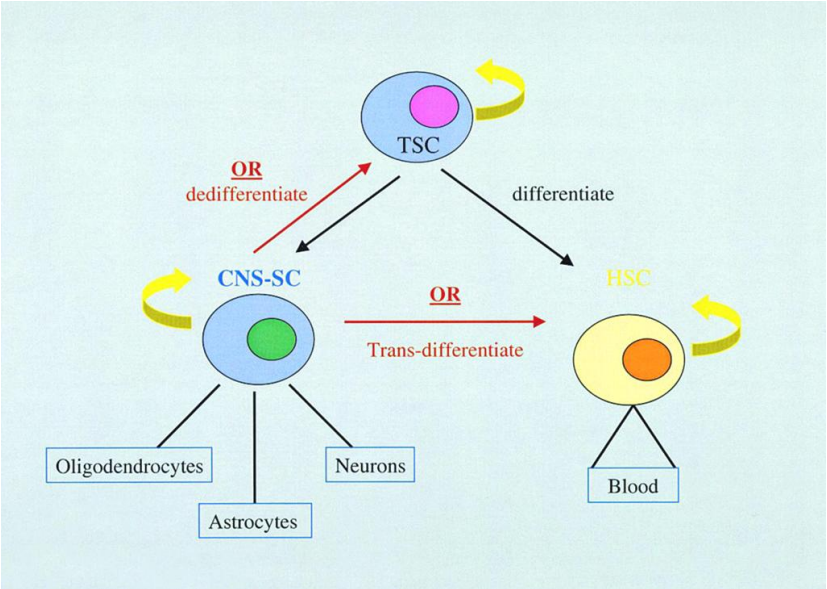



**Figure 1.10: Trans-differentiation stem cell kinetics for the formation of haematopoietic stem cells (HSC). Central nervous system stem cells (CNS-SC) shown to form HSC by trans-differentiation, or de-differentiation into an earlier totipotent stem cell (TSC) and differentiating into HSC (Weissman 2000).**

## 1.8. Stem Cell Differentiation

Maintenance of stem cell potential by signalling factors is vital for regulation of tissues. The differentiation of stem cells to a specialised cell type is equally an important aspect of tissue development and tissue regulation. Environmental factors

that influence stem cell differentiation include environmental forces, oxygen tension, cell-cell and cell-matrix signalling factors such as cytokines and growth factors, with scaffolds (either synthetic polymer or natural scaffolds) that also aid tissue development into their correct *in vivo* 3-dimensional (3D) configuration (Blain et al. 2003; Discher et al. 2009; Hung et al. 2011b; Wang et al. 2011; Zambonin and Grano 1995). Studies have shown DPSCs with dentine matrix protein-1 (Dmp-1), a NCP which induces odontoblast-like differentiation and is a nucleator for mineral formation (He et al. 2003; Narayanan et al. 2001), when cultured together in collagen gel scaffolds and placed in a perforated dentine slice, demonstrated new tissue formation and angiogenesis in the dentine slice. Culture with only DPSCs or Dmp-1, had only a few blood cells present with a few nucleated cells (suspected to be fibroblasts) with no considerable matrix production. (Prescott et al. 2008). This demonstrates the influence of extracellular signalling factors which play a role in inducing stem cell differentiation.

Another factor that induces differentiation is mechanical activity, such as the application of pressure and shear forces. This is converted to chemical stimulation (mechanotransduction) and encourages gene expression upregulation, such as during cartilage tissue development by differentiation of MSCs into chondrocytic cells with regular force application (Blain et al. 2003). Oxygen tension also influences cell differentiation, where MSCs in hypoxic conditions promote osteogenic differentiation and inhibits adipogenic differentiation (Hung et al. 2011b). However, with so many factors that can affect stem cell differentiation, it is stressed that differentiation is a tightly regulated process.

Organised tissue formation is a highly orchestrated process and over expression of developmental genes can lead to tumour formation. With dental tumours (odontoma) they are known to over express genes including LIM-homeobox 8 (*lhx8*) which is involved with regulating tooth morphogenesis. Sox2 and nestin which are normally involved with early tissue development are also known to be over expressed (Kim et al. 2011b; Song et al. 2009). Understanding stem cell differentiation and the factors/cues that govern its differentiation is important for manipulation of stem cell behaviour and formation of desired cell types for tissue engineering and regenerative therapy (Barry and Murphy 2004).

### 1.9. Niche Influence on Stem Cell Behaviour

Stem cells have been identified in small compartments within an assortment of tissues, commonly at highly vascularised sites. Regulation and directing differentiation to transit amplifying cells is determined by signals from its neighbouring cells and microenvironment, or its niche, vital for maintaining the "reserve" of stem cells for the replenishment, repair and homeostasis of tissues (Fuchs et al. 2004; Shi and Gronthos 2003; Spradling et al. 2001).

Stem cells in their *in vivo* conditions are more superior at maintaining their phenotype compared with *in vitro* culturing which does not provide all the necessary signal factors of its niche environment. Migration and cell attachment is suggested to be influenced by ephrin (Eph) signalling pathway, where cells can become affected by Eph-ligands or they themselves present the ligands to neighbouring cell Eph-receptors. EphB is suggested to have role in maintaining DPSC potential and within

its niche, by down-regulating differentiation, preventing cell dispersion and reducing focal adhesions until recruited to an injury site (Stokowski et al. 2007).

The influence from neighbouring progenitor cell populations in the same niche can also have a regulatory role. An example of this is the bone marrow, which is a well studied niche for haematopoietic stem cells (HSCs) and also MSCs, where they may co-exist and influence each other's behaviour, possibly by release of signalling factors or by cell-cell interactions (Fuchs and Segre 2000; Fuchs et al. 2004; Spradling et al. 2001). Previous studies have shown Notch signalling is upregulated during dentine injury, which causes differentiation of dental progenitors into perivascular and odontoblast-like cells (Lovschall et al. 2005). Notch is an evolutionary conserved transmembrane signalling pathway that occurs with direct contact between neighbouring cells.

The Notch ligands, delta-like and jagged (the human equivalents), are expressed on the surface of the ligand presenting cell, which bind to Notch receptors on the receiving cell's membrane. This causes a proteolytic cleavage, where the intracellular component of the Notch receptor traverses into the cell nucleus and modifies gene expression (Artavanis-Tsakonas et al. 1995; Oswald et al. 2001). This cell-cell interaction allows for cell coordination and tissue organisation, as part of tissue development or repair.

Extracellular signals are also another important factor of the niche. When foetal HSCs were transferred to an adult spleen, they then expressed adult globin genes, demonstrating the dominance of the environment which influences developmental

stage-specific gene expression (Geiger et al. 1998). When ESCs are transferred to a foreign environment *in vivo*, away from its original niche, it forms an uncontrolled multi-cellular mass, a teratoma, due to lack of signalling factors which regulate its pluripotency/development (such as Wnt, LIF, FGF, p53 expression, as mentioned earlier). This highlights the importance of correct cell signalling factors when using immature cells in tissue engineering (Fuchs and Segre 2000; Fuchs et al. 2004; Geiger et al. 1998).

The transfer of more mature adult stem cell into another tissue of the body, demonstrates its plasticity to differentiate into an unlike cell lineage (asymmetric cell kinetics, section 1.7). A study using murine brain stem cells, transferred to sub-lethally irradiated mice devoid of HSCs, demonstrated the ability of re-establishing the bone marrow stem cell population, most likely by de-differentiation of the neural cells. This illustrates the "reprogramming" of brain cells to a haematopoietic fate by adapting to their new niche and its new environmental cues (Bjornson et al. 1999; Fuchs and Segre 2000). Further understanding of niche signalling factors may be implemented for controlling adult stem cell behaviour, which is a useful attribute for tissue engineering and regenerative therapy.

### 1.10. Stem Cells of the Perivascular Niche

Stem cells reside in specific tissue niches that have good vascular systems, where multipotent progenitors are commonly found to be located around large blood vessels and microvasculature (Doherty et al. 1998; Farrington-Rock et al. 2004). Previous studies have shown Stro-1 isolated MSCs from bone marrow and dental pulp to be positive for cell surface pericytic markers including alpha-smooth muscle actin,

CD106 the vascular cell adhesion molecule-1 (VCAM-1), CD146 the melanoma cell adhesion molecule (MCAM) and 3G5 ganglioside component of the cell membrane. Each have distinct roles in cell attachment, migration and cell signal transduction, but are also characterised on pericyte cells (da Silva Meirelles et al. 2006; Gronthos and Zannettino 2008; Khan et al. 2010; Shi and Gronthos 2003). Stro-$1^+$ cells used to further select for a $CD106^+$ subpopulation, in an attempt for enrichment of potent progenitors, have stronger stem cell character *in vitro*, with higher proliferative ability and differentiation efficiency compared with just Stro-1 sorted cells (Gronthos et al. 2003). These results suggest enrichment selection for pericytic characteristics may produce a more multipotent progenitor population, making it a more valuable for tissue engineering purposes.

### 1.11. Isolation of Stem Cell Populations

Harvesting adult stem cells for tissue engineering purposes is based on identification of the stem cell characteristics. Somatic stem cells are identified by their ability to form clonal clusters (or colonies) called colony forming unit fibroblast (CFU-F) *in vitro*, which is a common characteristic of most stem cell populations including MSCs (Castro-Malaspina et al. 1980; Gronthos et al. 2000; Shi and Gronthos 2003; Weissman 2000). *In vivo* studies show matrix protein fibronectin is present at the distal end of the odontoblast layer through all the stages of dentinogenesis (Yoshiba et al. 1994). Fibronectin is an extracellular matrix component of the basement membrane (Ruch et al. 1995), which plays an important role in mediating adhesion of stem cells to the extracellular matrix, forming a part of the basement membrane and for the differentiation of odontoblasts and its orientation (Lesot et al. 1986; Nanci 2003; Tziafas et al. 2000). Members of the integrin family, such as $\alpha_5\beta_1$, have

a high affinity for the tripeptide motif Arg-Gly-Asp (RGD) found on ligands such as fibronectin (Hynes 1992). Immature cells have approximately twice the amount of $\beta_1$-integrins on the cell surfaces which functionally bind to fibronectin (Jones and Watt 1993). This preferential binding to fibronectin has provided a basis for isolating adherent progenitors by seeding harvested cells onto fibronectin coated plates for a short period, before removal of the unbound, less immature cells (Dowthwaite et al. 2004).

It is well known that multiple progenitors exist within tissues (Iohara et al. 2009; Waddington et al. 2009), and progenitor isolation based on adhesion by high levels of integrins may produce a very heterogeneous culture. This may be beneficial as the cells may influence each other as a closer representation of the niche environment (section 1.9), and may better maintain the stem cells in culture. However, this heterogeneity can render experimental study irreproducible as the different populations may have varying differential potentials. Clonally selected populations may be utilised, where a colony of cells consisting of 32 cells or more (5 PDs of a single cell clone) may be isolated and expanded in culture, although which is the best clone to isolate and expand remains uncertain (Dowthwaite et al. 2004; Jones and Watt 1993).

Another selection method includes fluorescence activated cell sorting (FACS) by flow cytometry, where cell fluorescence or the use of one or more antibodies can be used for the selection of cell markers in a mixed cell population, then automatically sorted into separate constituents of cell populations. For example, perivascular cells isolated using two perivascular cell markers, CD146 and platelet-derived growth

factor receptor-β (PDGFR-β), express MSC phenotypic surface markers and demonstrate mesenchymal differential potential (Schwab and Gargett 2007). An isolation method which is similar to FACS is magnetic activated cell sorting (MACS). This is also based on the use of antibodies as FACS, which utilises antibodies conjugated with a magnetic bead that bind to cells to be positively selected when passed through a magnetised column. MACS has been used on bone marrow cells for isolation of primitive MSCs of neural crest origin, where low-affinity nerve growth factor receptor (LANGFR) selected neural crest cells have been isolated by using an antibody for P75 nerve growth factor receptor and directed down mesenchymal lineages, demonstrating its multipotency as an adult stem cell (Quirici et al. 2002).

However, both MACS and FACS have their pros and cons. FACS does not only depend on surface antigens as it is based on cell fluorescence which can be transfected with fluorescent proteins, and multiple antigens can be targeted for sorting. However, FACS equipment is expensive, there is low through-put of positive cells, there is a chance of cross-contamination, and background or intrinsic cell fluorescence can affect the sorting procedure. With MACS, there is no need to pre-label cells with fluorescence and the instruments are inexpensive. The cons of using MACS is the use of a single antibody for labelling and sorting a cell population when there is no single definite stem cell marker determined and separation is based on the physical surface composition of cells, which may be compromised during cell harvesting (Gerashchenko 2011; Willis 2004).

The selection of progenitors has been shown with a variety of methods and each have produced stem cells of multipotent capacity; all are capable for use in tissue engineering studies. The method to use is determined by the researcher's needs, financial constraints and if specific stem cell characteristic is required (e.g. expression of Stro-1) to determine as a stem cell population.

### 1.12. Stem Cells of the Dental Tissue

There are a myriad of stem cell niches within the neural crest-derived dental organ, isolated populations include the pericytic progenitors within the adult tooth pulp (DPSCs), neural crest stem cells, stem cells from human exfoliated deciduous teeth (SHED), the immature cell population at the base of the dental papillae known as the stem cell of the apical papillae (SCAP), and also in the surrounding supportive tissue of the tooth, the periodontal ligament stem cells (PDLSC).

DPSCs were first isolated in 2000 by Gronthos and co-workers, where comparison of clonal populations to bone marrow stromal cells (BMSCs) show DPSCs are more proliferative and have a distinct expression profile. Both cell populations have similar fibroblast-like morphology, with DPSCs having slightly increased growth kinetics shown by its higher colony forming efficiency (CFE) and a higher percentage of proliferative cells.

Isolated DPSCs do not express haematopoietic markers (macrophage CD14, leukocyte CD45, common HSC antigen CD34) or typical cartilage, fat, muscle or nerve markers, but are positive for bone markers such as alkaline phosphatase (ALP), type I collagen, OP, ON, and OCN, also pericytic markers CD146 and α-smooth

muscle actin (Gronthos et al. 2000). Studies also show the presence of embryonic markers, such as Oct4, Nanog and SSEA, which is revealed to be in 80% of the isolated population when cultured up to 25 PDs (Dissanayaka et al. 2011; Guimarães et al. 2011; Kerkis et al. 2006). These markers are not expressed homogenously through all the DPSC cultures, illustrating heterogeneity of progenitors in the pulp and a preferential pulpal differentiation into mineralised tissues.

The expression of immature cell markers β1-integrin and FGF-2, and the lack of dentine markers indicate a population of undifferentiated cells (Gronthos et al. 2000; Hung et al. 2011a; Waddington et al. 2009). The expression of β1-integrins by the pulpal progenitors has facilitated DPSC isolation using fibronectin coated tissue culture plates.

The immature DPSC population with preferential binding to fibronectin are proliferative and positive for mesenchymal markers vimentin and Msh homeobox-1 (Msx1) (Waddington et al. 2009). Vimentin is a cytoplasmic filamentous protein, part of the cell cytoskeleton and found in melanoma (mesenchymal) cells (Gonzales et al. 2001; Li et al. 2010). Msx1 is vital for mesenchyme formation and development of limb tissue (Bensoussan-Trigano et al. 2011), where both markers are typically expressed by multipotent MSCs (Bensoussan-Trigano et al. 2011; Hu et al. 2011; Peters and Balling 1999; Tucker et al. 1998).

In the relevant induction medium, these DPSC can differentiate into the mesenchymal adipogenic, chondrogenic and osteogenic lineages, determined by histological staining (oil red O, toluidine blue and alizarin red S stains respectively)

and gene expression (Dissanayaka et al. 2011; Guimarães et al. 2011; Hung et al. 2011b), also odontogenic lineages by expression of dentin sialophosphoprotein (DSPP). This becomes cleaved to form DPP and DSP, and also Dmp-1 that coincides with positive alizarin S staining and alkaline phosphatase (ALP) expression as part of mineralisation (Kim et al. 2011a; MacDougall et al. 1997; Nam et al. 2011). DPSCs also have potential for neurogenic differentiation, by expression of neuron markers such as βIII-tubulin (Dissanayaka et al. 2011; Hung et al. 2011a).

It has been shown multiple progenitors reside in the dental pulp. $CD146^-$ cells sorted from the dental pulp produce an angiogenesis response with capillary formation with *in vitro* culture and with transplantation into an amputated pulp tooth cavity (Iohara et al. 2009), which demonstrates the multipotent potential of the distinctly separate population.

In a separate study, the presence of multiple progenitors is further illustrated with the selection of the more immature, neural crest stem cells from the pulp, where MACS isolated $LANGFR^+$ ($P75^+$) cells were able to differentiate into adipogenic, osteogenic and chondrogenic lineages (Waddington et al. 2009). This demonstrates the DPSCs are undifferentiated multipotent progenitors that contain some of the pluripotency markers and are able to differentiate into many lineages. As the pulp is a heterogeneous niche of multiple stem cell populations, the progenitors involved with dentine repair is difficult to determine. It is likely a proportion of the adult human pulpal progenitors may even include residual SCAPs that remain from tooth development.

The dental papilla is formed from the mesenchyme at the bell stage of tooth development and is the precursor to the dental pulp (section 1.3). Papillae cells are found in parallel to the basement membrane and epithelial cells, where the papilla cells differentiate into pre-odontoblasts. The randomly located pre-odontoblasts align to the basement membrane (which contains fibronectin) and form polarised odontoblasts for predentine secretion (Linde and Goldberg 1993). The base of the developing immature human tooth apex contains the immature papillae SCAP population, separated from the pulp by a cell rich zone (Huang et al. 2008; Sonoyama et al. 2008). Human apical papilla is less cellular and less vascularised when compared with the dental pulp, although is up to three times more proliferative with *in vitro* culture (Sonoyama et al. 2008), illustrating the SCAPs are a distinct cell population of the pulp.

Studies have shown SCAPs to be more immature, negative for odontogenic (DSP), osteogenic (BSP, OCN) and mineralisation (ALP) markers when compared with odontoblasts, although containing low gene expression of DSP, TGF-β and FGF gene markers. The MSC markers Stro-1 and CD146 were also expressed, illustrating the mesenchymal origins of the papillae cells, so it is unsurprising SCAPS have a similar multipotent differentiation potential as DPSCs. SCAPs can differentiate into the adipogenic (oil red $O^+$, lipid regulator peroxisome proliferator-activated receptor gamma isoforms 2 $(PPAR\gamma2)^+$, and fatty acid regulator lipoprotein lipase $(LPL)^+$), odontogenic, osteogenic (alizarin $red^+$) and neurogenic (β-III $tubulin^+$ and $Nestin^+$) lineages, where the ability to form the neurogenic lineage supports the neural crest origins of the papillae (Ding et al. 2010; Gronthos et al. 2002; Sonoyama et al. 2008).

With the rodent incisors, continuously erupting incisors are a well-known known characteristic (Catón and Tucker 2009; Smith and Warshawsky 1976). The continuous growth of the incisors is thought to be due to a large stem cell niche at the cervical loop of the tooth apex, the rodent equivalent of the SCAPs known as the apical bud cells (ABCs) (Ohshima et al. 2005). These cells are also proliferative and are able to differentiate into mesenchymal lineages and a study has even differentiated the ABCs into ameloblastic cells (Fang et al. 2009).

Although the differential potential of ABCs has not been fully explored yet, the similarity of the niche to SCAPs suggests a similar multipotent differentiation potential. SCAP/ABC cells are a distinct multipotent neural crest-derived progenitor population, shown to be more immature and proliferative than the DPSCs population, although capable of the same differentiation capacity.

In comparison to the human adult dental pulp, the deciduous tooth pulps also contain a distinct progenitor cell population known as the SHED cells. These cells are similar to SCAPs as they also are more immature and proliferative than DPSCs (Huang et al. 2009), and expresses MSC markers Stro-1 and CD146. Culture in mineralising medium produces calcium deposits, as shown by positive alizarin red staining, and also induces osteogenic and odontogenic gene markers which include Runx2, OCN and DSPP (Miura et al. 2003; Shi et al. 2005). The SHED cells also are able to differentiate into adipogenic and neurogenic lineages with expression that include LPL, PPARγ2, β-III tubulin and Nestin, which suggests again a neural crest origin (Miura et al. 2003; Wang et al. 2010). These SHED cells have similar capacity for differentiation as DPSCs and SCAPs, although are more immature than the DPSCs

as like SCAPs, but SHED cells are more easily accessible from deciduous teeth, which is an advantage for its accessibility for tissue engineering purposes.

Around the tooth, the supportive connective tissue also contains a progenitor population as PDLSC. The periodontal ligament (PDL) is a soft fibrous tissue between the root cementum and the surrounding alveolar bone socket (Shimono et al. 2003). The PDL is a highly vascularised tissue containing a heterogeneous cell population ($ALP^+$ and $ALP^-$) and with varying levels of cell commitment. PDLSCs migrate from perivascular sites to the cementum and alveolar bone and differentiate into cementoblasts/osteoblasts, shown by expressing typical markers such as Runx2, OCN, BSP and positive for alizarin red staining and ALP activity (Fujii et al. 2008; Gay et al. 2007; Gould et al. 1980; Ikeda et al. 2010; Murakami et al. 2003; Seo et al. 2004).

PDLSCs express a variety of MSC markers, including Stro-1 and CD146, two of the common MSC markers which are also found with stem cells of the dental pulp. With this mesenchymal property, it is unsurprising the PDLSCs are able to differentiate into adipocytes shown with oil red O positive staining, also $LPL^+$ and $PPAR\gamma2^+$ gene expression (Seo et al. 2004). However, the PDLSCs also have higher levels of scleraxis expression compared with DPSCs, scleraxis is typically expressed in tendon cells (Brent et al. 2003), which suggests of a distinct mesenchymal progenitor population from the pulpal progenitors (Fujii et al. 2008; Seo et al. 2004). The PDLSC are similar to the progenitors of the pulp, they are multipotent and able to differentiate into mesenchymal tissues, but are a distinctly different population of progenitors involved with cementum and PDL repair.

### 1.13. Dental Pulp Stem Cells in Dentine Repair

Within the dental niche, DPSCs naturally remain quiescent within its normal microenvironment, where cytokines from the surrounding microvasculature network maintain the DPSCs in an undifferentiated state (Nishikawa and Osawa 2007; Stocum 2001; Tecles et al. 2005). The tooth is able to detect injury (environmental stimuli) to its primary and secondary dentine, whether from attrition, abrasion, erosion, trauma, caries or by restorative procedures. Its response is to increase secretion of the protective tertiary dentine matrix at the affected foci at the dentine-pulp interface in order to protect its delicate pulp from pathogenic invasion (Smith et al. 1995; Tecles et al. 2005).

Mild injury (e.g. mild caries) causes existing odontoblasts to upregulate dentine secretion as reactionary dentinogenesis. Severe injury (e.g. dental fractures) and odontoblast death results in reparative dentinogenesis, where DPSCs divide by mitosis before migrating to the subodontoblast layer under the chemotactic stimuli. After a second mitosis the progenitors differentiate into odontoblast-like cells for the synthesis and secretion of dentine matrix, forming a discontinuity of tubular structure and reduction of dentine permeability (Fitzgerald et al. 1990).

Following dentine injury bacterial acids secreted by invading pathogens and proteolytic enzymes from the host system causes the release of DMPs from the dentine layer (Dung et al. 1995). Restorative materials, such as calcium hydroxide ($Ca(OH)_2$) used for pulp capping, gradually release hydroxyl ions which raise the local pH. This induces several outcomes; it becomes bactericidal, causes chemical

damage to the existing odontoblasts and also solubilises the DMPs (Goldberg and Smith 2004; Graham et al. 2006; Javelet et al. 1985; Kardos et al. 1998).

With *in vitro* studies, $Ca(OH)_2$ is shown to be less efficient in extracting NCP, compared to ethylenediaminetetraacetic acid (EDTA), which acts as a demineralising and chelating agent (Graham et al. 2006). However, the NCPs may naturally exist as functional aggregates (supramolecular complexes) in the dentine, and solubilisation may affect its functional activity (Butler and Ritchie 1995).

In the presence of DMP, the existing odontoblasts undergo reactionary dentinogenesis for secretion of tertiary dentine. With necrosis of the odontoblasts, DPSCs migrate from the dental pulp and differentiate into odontoblast-like cells, positive for mineralisation and odontogenic markers such as DSPP which forms DPP and DSP (Begue-Kirn et al. 1992; Fuchs and Segre 2000; Goldberg and Lasfargues 1995; Gronthos et al. 2000; Lesot et al. 1986; Shi and Gronthos 2003; Shi et al. 2001).

The initiating molecules for reactionary and reparative dentine are not clear, but are thought to be similar and may interact in a more complex manner (synergistic association) during reparative dentinogenesis. This highlights the benefits of using a cocktail of cytokines/growth factors in DMP for study of dentine repair (Bègue-Kirn et al. 1994; Murray et al. 2000; Ruch et al. 1995; Sloan and Smith 1999; Smith et al. 1994; Tziafas et al. 2000).

The use of tooth slice organ cultures has been used for investigating reactionary dentinogenesis after application of cavity preparations. The tooth slices conditioned with EDTA (or $Ca(OH)_2$) show an intact dentine layer is an important factor that affects the magnitude of the reactionary dentine response. The solubilisation of the existing bio-active molecules, particularly TGF-β, diffuses down the dentine tubules which stimulates odontoblast reactionary dentinogenesis and dentine bridge formation (Duque et al. 2006; Graham et al. 2006; Tziafas et al. 2000).

Studies reveal direct DMP implantation onto exposed dental pulps induces a strong reparative dentinogenesis response, with formation of new odontoblast-like cells at the pulp surfaces and secretion of tertiary dentine (Smith et al. 1990). These results collectively highlight the matrix components contain vital morphogenic proteins for upregulating odontoblast dentine secretion and attracting progenitors for repair and regeneration.

### 1.14. Aims

The overall aim of this thesis is to isolate and characterise an immature DPSC population that has multipotent differential potential for use with studying the effects of DMP. Specific DMP effects on cell viability, proliferation, apoptosis, differentiation and migration will be investigated. A preliminary study for localising DPSCs in an established *ex vivo* tooth slice model will allow monitoring cell behaviour *in situ*. These aims will be achieved by:

- Characterisation of human and rodent DPSCs isolated by fibronectin preferential selection and neural crest P75 MACS.

- Determining the differential potential of isolated DPSCs cultured in adipogenic, osteogenic and chondrogenic induction medium then analysed by gene expression and histology.

- Culturing DPSCs in DMP, assessing their vitality by its gene expression and utilising MTS and caspase assays. Cell proliferation will be examined by counting stained cells and differentiation effects determined by gene expression. DMP migratory effects on DPSCs will be determined by Boyden chamber experiments.

- Staining DPSCs with a fluorescent tracker dye and microinjection in a tooth slice and visualised with UV microscopy and immunohistochemisty.

# Chapter 2:

# Isolation of Distinct Populations of Dental Pulp Progenitors

## 2.1. Introduction

The heterogeneous population of cells in the dental pulp includes progenitors that migrate towards a dentine injury site as part of reparative dentinogenesis (Smith *et al.*, 1995, Tziafas *et al.*, 2000). Compared with bone marrow stromal cells, clonal populations of DPSCs were shown to be more proliferative with a different expression profile (Gronthos *et al.*, 2000). The multiple stem cell niches in the pulp include perivascular cells, MSCs, fibroblast-like cells or can perhaps be a unique population of DPSCs, all shown to lack haematopoietic markers (Gronthos *et al.*, 2002, Waddington *et al.*, 2009). However, the progenitor cells required for successful tooth regeneration and mineralisation is yet to be identified (Fitzgerald *et al.*, 1990, Gronthos *et al.*, 2000, Shi *et al.*, 2005, Sloan & Smith, 2007).

Characterisation of DPSCs show expression of putative MSC and pericytic markers which include Stro-1, Msx-1, CD31, CD44, CD146 and CD105 (Kerkis *et al.*, 2006, Patel *et al.*, 2009, Shi *et al.*, 2005, Shi & Gronthos, 2003, Waddington *et al.*, 2009). Presence of neural crest markers such as LANGFR suggested a niche of ectomesenchymal cells, presumed to be derived from the first branchial arch of the neural crest during early development (Deng *et al.*, 2004, Smith *et al.*, 2005, Waddington *et al.*, 2009). DPSCs express early developmental markers of the Notch signalling pathway, which were suggested to be associated with injury response and control of progenitor cell differentiation (Lovschall *et al.*, 2005, Sloan & Waddington, 2009, Waddington *et al.*, 2009). The immature DPSCs were also shown

to express embryonic markers Oct4, Nanog and stage-specific embryonic antigens (SSEAs) for at least 25 passages in culture, along with the expression of multipotent MSC markers. Expression of Oct4 occurred in only 80% of harvested pulpal cells, providing further evidence of a heterogeneous stem cell niche (Kerkis *et al.*, 2006).

The progenitor heterogeneity has an effect on the rate of odontogenesis and mineralisation as some progenitors may be more committed than others (Patel *et al.*, 2009, Yang *et al.*, 2007). The hierarchy of multiple stem cell niches which include committed progenitors in the pulp can affect the dentine repair response. Isolation of distinct populations of stem cells provided insight into the dynamics of the multiple stem cell niches (Scadden, 2006, Sloan & Waddington, 2009, Tecles *et al.*, 2005, Waddington *et al.*, 2009). More popular choices of selection have been FACS and MACS which are based on the selection for specific surface antigens (Gay *et al.*, 2007, Jones *et al.*, 2002, Miura *et al.*, 2003, Seo *et al.*, 2004, Waddington *et al.*, 2009, Yang *et al.*, 2007).

A recent isolation method utilises the high number of functional integrin receptors of the immature progenitor cells that preferentially bind to the ligand fibronectin, which is naturally present in the dentine matrix (Dowthwaite *et al.*, 2004, Hynes, 1992, Jones & Watt, 1993, Waddington *et al.*, 2009). The use of a single marker or methodology for progenitor selection may isolate only one of the many populations within the pulp. Previous work has shown the $CD146^{-}$ side-population of the dental pulp sorted by flow cytometry demonstrated multipotent characteristics (Iohara *et al.*, 2009), which supports the theory of multiple progenitor populations in the dental pulp.

This chapter aims to isolate distinct populations of progenitors from the dental pulp of both human and rodent tissues, where cell maturity will be determined by their expression of multipotent and pluripotent gene markers. Two isolation methods for progenitor selection will be fibronectin preferential adherence and sorting of P75 neural crest cells. The different isolated populations will then be compared alongside its negatively selected side populations, where the growth kinetics and expression profile for multipotent and pluripotent stem cell markers would be observed. These results will collectively demonstrate whether different isolated populations are distinct progenitors, and at the same time establish a DPSC population for future experiments and determining its differential potential and behaviour.

## 2.2: Materials and Methods

### 2.2.1. Cell Sources

Human impacted third molars from anonymised adults (typically 18-35 years old) were acquired from the School of Dentistry, Cardiff University (UK) with informed patient consent as required under the ethical conditions approved by the South East Wales Research Ethics Committee of the National Research Ethics Service (NRES), UK. A copy of the patient information sheet and consent form is included at the appendix.

The 28 days old male Wistar rats (*Rattus norvegicus*) used were supplied by Harlan Laboratories (UK). The animals were handled by the Biomedical Services, Cardiff University (UK) and were sacrificed by carbon dioxide ($CO_2$) asphyxiation in accordance with Schedule 1 of the United Kingdom Home Office guidelines.

### 2.2.2. Culture Medium

Progenitor cells were cultured in alpha-modification Minimum Essential Medium (αMEM) pre-supplemented with ribonucleosides and deoxyribonucleosides (Gibco, Invitrogen, UK). This was supplemented similar to previous literature medium components using high serum content for culture expansion (Gronthos et al. 2000; Vater et al. 2011; Waddington et al. 2009). The medium was supplemented to a final concentration of 20% heat-inactivated foetal calf serum (hi-FCS) that was E.U. approved (Invitrogen, UK), 4 mM L-glutamine (Gibco, UK), 100 μM L-ascorbate 2-phosphate (Sigma-Aldrich, UK) and antibiotics/antimycotics containing 100 units/mL penicillin G sodium, 0.1 μg/mL streptomycin sulphate and 0.25 μg/mL amphotericin (Gibco, UK). Medium was pre-warmed to 37 ℃ prior use.

### 2.2.3. Cell Maintenance

Cells were cultured in tissue culture plates or filter capped flasks (both Greiner Bio-One, UK) at 37 ℃ with 5% carbon dioxide ($CO_2$) until 80-90% confluence for passaging. Passaging was completed by washing the cells with 0.1 M phosphate buffered saline (PBS, pH 7.4) before it was treated with pre-warmed 0.05% trypsin (Gibco). Incubation was carried out at 37 ℃ for 5 mins or until the cells had become detached from the tissue culture plate/flask.

The enzyme was then inactivated and the cells collected by the addition of culture medium containing FCS then centrifuged (Precision Durafuge 200, Thermo Scientific, UK) at 400 ×g for 5 mins. The resulting pellet was resuspended in 1 mL culture medium and cell numbers assessed by diluting in 0.4% trypan blue vitality stain (Sigma-Aldrich, UK). The stained cells were viewed in a Neubauer haemocytometer with an inverted phase-contrast light microscope (Olympus CK2 with a Nikon Coolpix 4500 camera attachment), before cell counting and seeding into a tissue culture plate/flask, typically at $5 \times 10^3/cm^2$.

### 2.2.4. Extraction of Dental Pulp Tissue

#### 2.2.4.1. Human Dental Pulp Extraction

Three or sometimes four human third molars (wisdom teeth) from the same patient were kept hydrated in antibiotic treated αMEM and all were processed within an hour after harvesting. Samples were processed in a tissue culture laboratory within routinely sterilised class II tissue culture hoods. The human tissues were handled by wearing personal protective equipment such as double-gloves and safety spectacles as protection from potential infectious diseases to the handler. The soft tissue surrounding the tooth

was cleared away using a scalpel before it was submerged briefly in 70% industrial methylated spirits (IMS) for 10 s for sterilisation. A diamond-edged bone saw was used to cut a groove laterally along the tooth crown and root to the dentine layer, so the tooth could be easily split using a chisel and hammer. The exposed pulp tissue was gently extracted using non-toothed pointed forceps to minimise damage, the pulps were pooled together and kept hydrated in antibiotic treated αMEM.

#### 2.2.4.2. Rodent Dental Pulp Extraction

Four rats were bathed for 1 min in 70% IMS for sterilisation. Incisions were made along the soft mucosa of the mouth to allow easier access to the teeth, then the connective tissue surrounding incisors was cut using a scalpel and the incisors extracted using forceps. Pulp tissue was obtained by teasing out the contents from the exposed end of the incisors or by dividing each incisor longitudinally to reveal the pulp tissue. All pulp tissue was pooled and kept hydrated in antibiotic treated αMEM.

### 2.2.5. Production of a Single Cell Suspension from Dental Pulp Tissues

The extracted pulp tissue was quickly shredded using a scalpel and immediately treated to pre-warmed 4 mg/mL collagenase/dispase (Roche Applied Science, UK) for incubation at 37°C for 1 hr with periodic shaking. The swift processing minimises potential inflammatory responses by the pulp tissue. The dissociated cells were passed through a 70 μm cell strainer (BD Falcon, UK) and 5-10 mL medium containing serum were also passed through the strainer to produce a single cell suspension. The cells were centrifuged at 400 ×g for 5 mins before the supernatant was discarded and the cell pellet resuspended with fresh medium, then centrifuged again that ensured complete removal

of the enzymes. The cells were then resuspended in culture medium for culturing, or serum-free medium for cell isolation procedures.

### 2.2.6. Fibronectin Preferential Selection

A concentration of 10 μg/mL fibronectin derived from human plasma (Sigma-Aldrich, UK) was reconstituted in 0.1 M $PBS^{+}$ (PBS containing 1 mM $Ca^{2+}$ and 1 mM $Mg^{2+}$, pH 7.4). This was used to pre-coat the base of 10$cm^2$ 6-well plates (Greiner Bio-One, UK) at 1 mL/well at 4°C overnight. The excess was aspirated prior to the addition of cells.

A single cell suspension resuspended in 1 mL serum-free medium was seeded into the pre-coated wells at 4 $\times 10^3$ cells/$cm^2$ for 20 mins at 37°C. The cells that remained adherent were the fibronectin adherent (FNA) population and the non-adherent (NA) population of cells were pippetted into a different well. Both populations were culture expanded into larger size 75$cm^2$ T75 flasks (Starstedt, Germany).

### 2.2.7. Magnetic Activated Cell Sorting for P75 Cells

Magnetic activated cell sorting (MACS) protocols followed the MiniMACS kit instructions (Miltenyi Biotec Ltd., UK) with all centrifugation steps performed at 4℃ where possible and at 300 ×g. A single cell suspension of $2\times10^6$ pulp cells in serum-free medium was centrifuged and all the supernatant discarded. The pellet was resuspended with the relevant monoclonal anti-P75 antibodies for the human and rodent dental pulp cells (sections 2.2.7.1. and 2.2.7.2. respectively) and sorted with magnetised MACS Columns (Miltenyi Biotec Ltd, UK) for magnetic separation of $P75^{+}$ cells (section 2.2.7.3).

### 2.2.7.1. Human Dental Pulp Cell P75$^+$ Labelling

For the human P75$^+$ (hP75$^+$) selection, the cell pellet was resuspended in 80 µL MACS buffer (PBS pH 7.2, 0.5% BSA (Fisher Scientific, UK) and 2 mM EDTA (Sigma-Adrich, UK) which was filter sterilised and degassed), 10 µL fragment crystallisable receptor (FcR) blocking buffer and 10 µL mouse anti-CD271 (P75) IgG antibody with allophycocyanin (APC) conjugate (both Miltenyi Biotec Ltd, UK). This was incubated at 4 ℃ for 10 mins with regular gentle agitation. The cells were then washed twice with 1 mL MACS buffer and centrifuged for 10 mins, removing all supernatant. The pellet was resuspended in 70 µL MACS buffer, 10 µL FcR blocking buffer and 20 µL anti-APC microbeaded antibody. This was incubated at 4 ℃ for 15 mins with regular gentle agitation. The cells were then washed twice by adding 1 mL of MACS buffer and centrifuged for 10 mins, removing all supernatant and the pellet resuspended in 500 µL of MACS buffer.

### 2.2.7.2. Rodent Dental Pulp Cell P75+ Labelling

For the rodent P75$^+$ (rP75$^+$) selection, the dental pulp cells were resuspended in 200 µL (concentration 10 µg/mL in 0.5% BSA) of mouse monoclonal anti-192-IgG$_1$ (P75) antibody (Santa Cruz Biotechnology Inc, Germany). This was incubated at 4 ℃ for 30 mins with regular gentle agitation before washed twice by adding 3 mL of MACS buffer and centrifugation at 300 ×g, aspirating and discarding the supernatant after each spin. The pellet was then resuspended with 40 µL anti-mouse IgG$_1$ antibody with microbead conjugate (Miltenyi Biotec Ltd, UK) and 160 µL MACS buffer. This was incubated at 4 ℃ for 15 mins with regular gentle agitation before it was washed twice with 3 mL MACS buffer and centrifugation at 300 ×g, the supernatant was aspirated and discarded after each spin. The final pellet was resuspended in 500 µL MACS buffer.

### 2.2.7.3. Magnetised Column Sorting of $P75^+$ Cells

A "MS" Column was placed into the MACS Separator magnet (Miltenyi Biotec Ltd, UK) and 500 µL of MACS buffer was passed through the column to rinse and prepare the column. The microbead labelled P75 cells were then passed through the columns and the negative flow-through was collected into 15 mL conical tubes (Greiner Bio-One, UK). The column was washed $3 \times 500$ µL of MACS buffer, adding each wash when the column reservoir was emptied.

To collect the $P75^+$ cells the column was removed from the MACS separator magnet and placed into a different 15 mL conical tube. 1 mL of MACS buffer was added into the column and the $P75^+$ cells were flushed out by firmly pushing the supplied plunger into the column. The collected $P75^+$ and $P75^-$ cell fractions were then centrifuged for 5 mins and cell pellets were resuspended in 200 µL culture medium. Cell numbers were determined with trypan blue staining (as shown in section 2.2.3.) and the cells seeded onto 96-well plates (Greiner Bio-One, UK) for culture expansion to $75cm^2$ T75 flasks (Starstedt, Germany).

### 2.2.8. Fluorescence Imaging of the Actin Cytoskeleton

The actin cytoskeleton of rodent cells was stained with phalloidin-FITC to determine cellular morphology. The cells were cultured on top of untreated 13 mm diameter sterile glass cover-slips (Raymond A Lamb, Thermo Scientific, UK) and placed on the base of 10 $cm^2$ 6-well plates (Greiner Bio-One, UK), the cells were then grown to 50% confluency and washed with PBS. The cells were fixed with 1 mL/well 4% paraformaldyhyde (PFA) and incubated for 10 mins at room temperature before the PFA was aspirated and washed twice with 1 mL/well PBS at 5 mins each. The PBS was then

aspirated before the cells were treated with 1 mL/well 0.3% Triton-X 100 (Boehringer Mannheim, Germany) for 30 mins with gentle shaking at room temperature. This was then aspirated to wash the cells twice with tris-buffered saline (TBS, pH7.5) and the cells were blocked at 1 mL/well with 1% fraction V bovine serum albumin (Fisher Scientific, UK) in TBS (1% BSA-TBS) for 1 hr at room temperature with gentle shaking.

The blocking buffer was removed before the addition of 1 mL/well 20 μg/mL phalloidin-FITC (Sigma-Aldrich, UK) in 1% BSA-TBS and incubated for 1 hr at 4 ℃ with gentle shaking and maintained in darkness. The stain was then aspirated and cells washed twice with 1 mL/well TBS at 5 mins each. A nuclei counter-stain, bisBenzimide (Sigma-Aldrich, UK), was diluted in 1% BSA-TBS and added at 1 mL/well at a 2.5 μg/mL concentration. This was incubated in the dark for 30 mins at 4°C with regular agitation before the wells were washed 3× with TBS.

The glass cover-slips in the wells were lifted off using forceps and mounted on glass slides in Fluorsave reagent (Calbiochem, Merck Chemicals, Germany) and allowed to set overnight in the dark at 4 ℃. The images were then captured using an ultra violet (UV) microscope: Olympus AX70 with Digital Eclipse DXM1200 digital camera attachment. The images were captured using Automatic Camera Tamer (ACT-1) control software (Nikon Digital) at 373 nm/456 nm (FITC) and 490 nm/520 nm (bisBenzimide).

### 2.2.9. Time-Lapse Microscopy

A clonal population of hFNA cells were cultured at $5 \times 10^3$ cells/cm$^2$ in a 10cm$^2$ 6-well plate (Greiner Bio-One, UK) for time lapse microscopy. Microscopy environment

conditions were maintained at 37 ℃ with 5% $CO_2$ with a Solent incubation chamber (Solent Scientific Ltd, UK) and the cells viewed at ×10 magnification using a Zeiss Axiovert 200 inverted microscope (Carl Zeiss Ltd, UK) with a ORCA-ER camera attachment (Hamamatsu Photonics KK, Japan). Cells recorded for time-lapse were cultured for 1-3 days, time-lapse was stopped to check recording, then recording was resumed from 3-5 days where a time-lapse video was captured using the MetaMorph image analysis software (Molecular Devices, USA).

### 2.2.10. Colony Forming Efficiency between Fibronectin Adherent and Non-Adherent cells

After the initial 24 hrs in culture the total number of cells adhered to the bottom of the culture plate were manually counted using light microscopy. The numbers of colonies were counted at 3, 6, 10 and 12 days from initial culture to determine colony forming efficiency (CFE). A colony was determined as ≥32 cells (Jones and Watt 1993) which is a representation of five population doublings (PDs) from a single adult stem cell. The CFE was calculated as:

*CFE = (number of colonies on day x) / (24 hr total cell count)*

The CFE for FNA and NA populations at each time point was collated (n= ≥3) into the computer statistical software GraphPad Instat (USA) which calculated the average and determined the standard error of mean (SEM). A total of 30 wells each was analysed for the rodent rFNA and rNA, with n = 6 for $rP75^{+}$ and n = 3 for $rP75^{-}$. For the human cells, hFNA and hNA had n = 10 for both sets. The CFEs between FNA and NA populations and $P75^{+}$ and $P75^{-}$ populations were used for comparison.

### 2.2.11. Isolating Clonal Populations of Cells

The undersides of each culture plate of colonies selected for clonal expansion were marked for easy identification. The wells were washed with PBS pH7.2 and the colonies were enclosed within a cloning ring (the cut end of a pipette tip) sealed with sterile paraffin wax. 50 µL of pre-warmed trypsin was gently added to the colony enclosed within the cloning ring. This was incubated at room temperature for 5 mins or when the cell colony had dissociated. Using 200 µL of culture medium the cells within the cloning ring were gently pippetted up and down to aid cell detachment before being transferred into a sterile 1.5 mL eppendorf (Eppendorf, UK). They were then centrifuged at 400 ×g for 10 mins before being counted with trypan blue (section 2.2.3) and seeded into 96-wells for culture expansion, maintaining the seeding density at $5 \times 10^3/cm^2$ at each passage.

### 2.2.12. Determining the Population Doublings of Cells

Cell cultures at each passage had the cell numbers obtained and cell numbers reseeded recorded. The data was entered into the formula below to determine the population doublings (PD):

$$PD = (\log_{10}(\text{total cell count obtained}) - \log_{10}(\text{total cell count re-seeded})) / \log_{10}(2)$$

The PDs were qualitatively compared as statistical comparison is not possible as each PD is of a single population and the date of the culture passage is usually different for each cell population.

### 2.2.13. Gene Markers of Multipotency and Pluripotency in DPSCs

Reverse transcription - polymerase chain reaction (RT-PCR) was carried out on the heterogeneous whole pulp tissue, heterogeneous FNA/NA cells (colonies allowed to merge), clonal FNA/NA cells and $P75^{+}/P75^{-}$ cell populations. RNA samples were acquired at early culture (passage 10 around 20 PDs) and later culture (passage 50 around 100 PDs), or as near as possible with culture time restraints.

Positive human controls consisted of 501mel (a human melanoma cell line kindly supplied by Dr. Lindsay Davies, Cardiff University, UK) and H9 (human embryonic stem cells kindly supplied by Dr. Nick Allen, Cardiff University, UK). For negative controls, water and RT-negative controls were used. The PCR was carried out with primers for mesenchymal markers, neural crest markers, embryonic markers, and early developmental markers with housekeeping gene β-actin. Details of the markers are shown in table 2.1 and table 2.2 for human and rat primer sequences respectively.

#### 2.2.13.1 Cell Lysis and Total RNA Extraction

Total RNA was extracted from cell cultures using spin columns of the RNeasy Mini Kit (Qiagen, UK) based on the manufacturer's protocols. Cells were initially cultured in $10cm^2$ 6-well plates (Greiner Bio-One, UK) until 90% confluence. Each well of cells was then lysed with 200 µL of Buffer RLT lysis buffer (Qiagen, UK) supplemented with 10 µL/mL of β-mercaptoethanol (Sigma-Aldrich, UK). The lysate was transferred to a QiaShredder column (Qiagen, UK) 600 µL at a time for centrifugation at 13,500 ×g for 2 mins (Spectrafuge 24D, Jencons, UK) to shear cellular components (including DNA) and separating cellular debris.

**Table 2.1: Human multipotent and pluripotent gene markers used for PCR amplification. β-actin was used as a housekeeping gene.**

| *Gene Product* | Human | | | |
|---|---|---|---|---|
| | ***Primer Sequence 5' - 3'*** | ***Annealing Temperature (°C)*** | ***Product Size (bp)*** | ***Source*** |
| CD105 (Endoglin)<br>*eng* | Forward: 5'-GAAGGGCTGCGTGGCTCAGG-3'<br>Reverse: 5'-CCTTCCAAGTGGCAGCCCCG-3' | 55 | 483 | Author's |
| CD146 (MCAM)<br>*mcam* | Forward: 5'-CGACAACGGGGTCCTGGTGC-3'<br>Reverse: 5'-CAGCGATAGCCGCCTCCTGC-3' | 55 | 321 | Author's |
| MSX-1 (Msh homeobox 1)<br>*msx1* | Forward: 5'-GAAGCCCGAGAGGACCCCGT-3'<br>Reverse: 5'-AGGCACCGTAGAGCGAGGCA-3' | 55 | 410 | Author's |
| LANGFR/P75/CD271<br>*ngfr* | Forward: 5'-CTGCAAGCAGAACAAGCAAG-3'<br>Reverse: 5'-GGCCTCATGGGTAAAGGAGT-3' | 55 | 310 | Author's |
| Slug<br>*snai2* | Forward: 5'-GAGCATACAGCCCCATCACT-3'<br>Reverse: 5'-CTCCCCCGTGTGAGTTCTAA-3' | 55 | 479 | Author's |
| Snail<br>*snai1* | Forward: 5'-GCGAGCTGCAGGACTCTAAT-3'<br>Reverse: 5'-CCAGGCTGAGGTATTCCTTG-3' | 55 | 441 | Author's |
| Twist<br>*twist1* | Forward: 5'-GTCCGCAGTCTTACGAGGAG-3'<br>Reverse: 5'-GATGCCTTTCCTTTCAGTGG-3' | 55 | 711 | Author's |
| hTERT<br>*tert* | Forward: 5'-CGGAAGAGTGTCTGGAGCAA-3'<br>Reverse: 5'-GGATGAAGCGGAGTCTGGA-3' | 55 | 145 | Mantripragada KK *et al* (2008) |
| hTR<br>*terc* | Forward: 5'-CTAACCCTAACTGAGAAGGGCGTA-3'<br>Reverse: 5'-GGCGAACGGGCCAGCAGCTGACATT-3' | 55 | 154 | Mantripragada KK *et al* (2008) |
| Oct4(a)<br>*pou5f1* | Forward: 5'-AGGAGTCGGGGTGGAGAG-3'<br>Reverse: 5'-CGTTTGGCTGAATACCTTCC-3' | 55 | 250 | Dr. L Davies (Cardiff University) |
| Nanog<br>*nanog* | Forward: 5'-CAAAGGCAAACAACCCACTT-3'<br>Reverse: 5'-CAGGACTGGATGTTCTGGGT-3' | 55 | 432 | Author's |
| Notch 1<br>*notch1* | Forward: 5'-CTACCTGTCAGACGTGGCCT-3'<br>Reverse: 5'-CGCAGAGGGTTGTATTGGTTCG-3' | 55 | 356 | Dr. L Davies (Cardiff University) |
| Notch 2<br>*notch2* | Forward: 5'-AAGCAGAGTCCCAGTGCCTA-3'<br>Reverse: 5'-CAGGGGGCACTGACAGTAAT-3' | 55 | 172 | Dr. L Davies (Cardiff University) |
| Notch 3<br>*notch3* | Forward: 5'-CAGTCGCCTGAGAATGATCAC-3'<br>Reverse: 5'-GAATGACCAGCAGCAAGACAG-3' | 55 | 195 | Dr. L Davies (Cardiff University) |
| Jagged 1<br>*jag1* | Forward: 5'-GACTCATCAGCCGTGTCTCA-3'<br>Reverse: 5'-CTGGGGAACACTCACACTCAA-3' | 55 | 190 | Dr. L Davies (Cardiff University) |
| Jagged 2<br>*jag2* | Forward: 5'-CTACAATGGTGGCATCTGTG-3'<br>Reverse: 5'-GCGATACCCGTTGATCTCAT-3' | 52 | 156 | Dr. L Davies (Cardiff University) |
| β-actin<br>*actb* | Forward: 5'-AGGGCAGTGATCTCCTTCTGCATCCT -3'<br>Reverse: 5'-CCACACTGTGCCCATCTACGAGGGGT -3' | 65 | 480 | Dr. XQ Wei (Cardiff University) |

**Table 2.2:** **Rodent multipotent and pluripotent gene markers used for PCR amplification. β-actin was used as housekeeping gene.**

| *Gene Product* | Rodent | | | |
|---|---|---|---|---|
| | *Primer Sequence 5' - 3'* | | *Annealing Temperature (°C)* | *Product Size (bp)* | *Source* |
| CD105 (Endoglin)<br>*eng* | Forward:<br>Reverse: | 5'-CGGTCTCCAGCTGCGGTGGTGGGCTCC-3'<br>5'-CACTGCCACCACGGGCTCCCGCTTGCT-3' | 62 | 896 | Author's |
| CD146 (MCAM)<br>*mcam* | Forward:<br>Reverse: | 5'-GCAGCGCCACGGGTGTGCCAGGAGAGG-3'<br>5'-CCCCACTGTGGTGCTTCTGGGCGGGCT-3' | 62 | 900 | Author's |
| MSX-1 (Msh homeobox 1)<br>*msx1* | Forward:<br>Reverse: | 5'-GGCGCCAAGCCCAAAGTGCCCGCTTCA-3'<br>5'-GAAAGGAGAGGCCGAACGCAGCAGGCG-3' | 62 | 622 | Author's |
| LANGFR/P75/CD271<br>*ngfr* | Forward:<br>Reverse: | 5'-CGCCCTGGGCTGATGCTGAATGCGAAG-3'<br>5'-CTGGATGCGTCGCAGGGCGGCTAAAAG-3' | 62 | 677 | Author's |
| Slug<br>*snai2* | Forward:<br>Reverse: | 5'-CACTCCCCTCTGCCCAGCGGCCTTTCT-3'<br>5'-GGCATGGGGGTCTGAAAGCTTGGGCTG-3' | 62 | 171 | Author's |
| Snail<br>*snai1* | Forward:<br>Reverse: | 5'-GCGAGCTGCAGGACGCGTGTGTGGAGT-3'<br>5'-CGGCAAAGGCACGGTTGCAGTGGGAGC-3' | 62 | 597 | Author's |
| Twist<br>*twist1* | Forward:<br>Reverse: | 5'-GTTAGGGCGCCCCGCTCCTCTGCTATT-3'<br>5'-GCGCAGGGCGGCAAATGCCTCGTTCAG-3' | 62 | 473 | Author's |
| TERT<br>*tert* | Forward:<br>Reverse: | 5'-GCTCCGGTTACACAGCAGCCCCTGGCA-3'<br>5'-GGTCCAGAGCACGCACACGCAGCACGA-3' | 62 | 752 | Author's |
| TR<br>*terc* | Forward:<br>Reverse: | 5'-CTCCGCCCGCTGTTTTTCTCGCTGACT-3'<br>5'-GCCACCGAACTCAGGGACCAGTTCCGT-3' | 62 | 300 | Author's |
| Oct4(a)<br>*pou5f1* | Forward:<br>Reverse: | 5'-GCCCACCTTCCCCATGGCTGGACACCT-3'<br>5'-GCAGGGCCTCGAAGCGGCAGATGGTTG-3' | 62 | 563 | Author's |
| Nanog<br>*nanog* | Forward:<br>Reverse: | 5'-GGGGATTCCTCGCCGATGCCTGCCGTT-3'<br>5'-GGGATACTCCACCGGCGCTGAGCCCTT-3' | 62 | 477 | Author's |
| Notch 1<br>*notch1* | Forward:<br>Reverse: | 5'-CGCGGGTGCGAGCGCAGTGAAGGAACG-3'<br>5'-GTGTGGGTGGCACGGCAGGCACAGCGA-3' | 62 | 837 | Author's |
| Notch 2<br>*notch2* | Forward:<br>Reverse: | 5'-CCCGCGCACGCTTTGCAGTGTCGAGGT-3'<br>5'-CTCCAGCCGTTCACGCACACGCAGCCG-3' | 62 | 929 | Author's |
| Notch 3<br>*notch3* | Forward:<br>Reverse: | 5'-GGTTGCCAGGCGCAAGCGAGAGCACAG-3'<br>5'-CATATCGGGCGGCCAAATGCAGGGCGG-3' | 62 | 560 | Author's |
| Jagged 1<br>*jag1* | Forward:<br>Reverse: | 5'-CAAGGCCAGCCGCGGCAACGACCGTAA-3'<br>5'-GCTTGGGACTGCAGCCCTGTCGGCATA-3' | 62 | 398 | Author's |
| Jagged 2<br>*jag2* | Forward:<br>Reverse: | 5'-CGCACAGACCGCACCCTCGCCTCCTTTA-3'<br>5'-CCGGGGCCGGCAGAACTTGTTGCAGGTG-3' | 62 | 678 | Author's |
| β-actin<br>*actb* | Forward:<br>Reverse: | 5'-TGAAGATCAAGATCATTGCTCCTCC-3'<br>5'-CTAGAAGCATTTGCGGTGGACGATG-3' | 56 | 155 | Gatto M *et al* (2008) |

### 2.2.13.2. Collection of Total RNA and DNase Digestion

The cell lysate was treated with 1:1 volumes of 70% molecular-grade ethanol (Sigma-Aldrich, UK) and mixed thoroughly by pipette action. The entire sample (600 µL at a time) including the precipitate of genomic material was then transferred into an RNeasy Mini Kit column (Qiagen, UK) and centrifuged at 10,000 ×g for 15 s. The flow-through was discarded and the column was washed with 350 µL of Buffer RW1 (Qiagen, UK) and centrifuged again at 10,000 ×g for 15 s.

The flow-though was discarded and the column was treated with a mixture of 10 µL of DNase I stock and 70 µL Buffer RDD (both Qiagen, UK). This was incubated at room temperature for 15 mins for on-column digestion to remove any contaminating genomic DNA. 350 µL of Buffer RW1 was added to the column and then washed by centrifugation at 10,000 ×g for 15 s. A further 2 × 500 µL of Buffer RPE was added and centrifuged for 1 min each time with the flow-though discarded after each spin.

The column insert was then transferred into an RNase-free 1.5 mL eppendorf (Epperndorf, UK) and 40 µL of diethylpyrocarbonate treated water (DEPC-$H_2O$) was added for eluting the RNA by centrifuging at 10,000 ×g for 1 min. The eluate was then pippetted back into the column and centrifuged again to maximise yield. Total RNA collected in the eppendorf was stored at -80°C.

### 2.2.13.3. Quantification of Total RNA

A NanoVue (GE Healthcare, UK) spectrophotometer was used to quantify total RNA. The NanoVue was standardised with 2 µL DEPC-$H_2O$ as a blank before using 2 µL of

RNA sample to quantify. The absorbance ratio at 260:280 nm between 1.7 and 2.2 indicated ideal RNA purity.

### 2.2.13.4. Complimentary DNA Generation by Reverse Transcription

Generation of complimentary DNA (cDNA) was performed using the Moloney murine leukaemia virus (M-MLV) reagents from Promega (UK). With a sterile 0.2 µL flat-cap tube (Thermo Scientific, UK), 1 µg of total RNA was treated with 0.5 µg of Random Primers (Promega, UK) with the addition of DEPC-$H_2O$ to adjust to a final volume of 15 µL. This mixture was then placed in a G-storm™ GS1 thermal cycler (Genetic Research Instrumentation Ltd, UK) and incubated at 70°C for 5 mins to unravel RNA.

The product was immediately chilled on ice before 5 µL of 5× M-MLV buffer was added along with 0.625 µL of RNasin, 1.25 µL of 10 mM dNTPs, 1 µL of M-MLV reverse transcriptase (all Promega, UK) and the addition of DEPC-$H_2O$ brought the final volume to 25 µL. The sample was gently mixed with a pipptette and placed into a thermal cycler at 25°C for 10 mins, 37°C for 1 hr, followed by 95°C for 5 mins. The newly generated cDNA was stored at -20°C.

### 2.2.13.5. Gene Amplification with Polymerase Chain Reaction

For a typical PCR reaction in a 0.2 µL flat-cap tube (Thermo Scientific, UK), 0.5 µL of cDNA (section 2.2.13.4) was added to a mixture of 5 µL of 5× Green GoTaq Flexi Buffer, 0.75 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs, 1.25 µL of 10 µM of forward primer, 1.25 µL of 10 µM reverse primer, 0.25 µL of GoTaq Flexi DNA

Polymerase (all Promega, UK) with the addition of DEPC-$H_2O$ to bring to a final volume of 25 µL.

The sample was placed in a thermal cycler at the typical conditions: initial denaturation at 94°C for 5 mins, then 40 cycles of denaturing, annealing and extension at 94°C for 1 min, 62°C for 30 s and 72°C for 1 min respectively. After the 40 cycles a final extension at 72°C was for 10 mins before being held at 4°C prior agarose gel analyses.

### 2.2.13.6. Agarose Gel Analysis of PCR Products

A 1.5% agarose gel was prepared by adding 0.75 g of UltraPure Agarose powder (Invitrogen, UK) to 50 mL of 1× TAE buffer (40 mM Tris acetate and 1 mM EDTA, pH 8.5) in a sterile beaker. This was heated in a microwave at 10 s intervals until the agarose had completely dissolved before 3 µL of 10 mg/mL molecular grade ethidium bromide (Promega) was added.

This mixture was then poured into a gel electrophoresis casting tray with an appropriate well comb and was allowed to set, then transferred into an electroporesis tank (GeneFlow, UK) which contained 1 × TAE buffer. 5 µL of 1 kb DNA ladder (Promega, UK) was pippetted to the first well and then 5 µL of the PCR samples were pipetted into the following wells. This was ran at 90V until the sample dye reached a suitable distance for viewing the separated PCR products with the use of a Bio-Rad Universal Hood and Quantity One GelDoc software (Bio-Rad, Italy).

### 2.2.14. Statistical Analysis

The average values and standard error of mean (SEM represented as ±) were determined using computer statistical software GraphPad Instat (USA). Samples were compared using analysis of variance (ANOVA) but when the internal test determining ANOVA was not viable (non-parametric), a post-hoc two-tailed Mann-Whitney statistical test was performed. A post-hoc Tukey-Kramer multiple comparisons test was performed when ANOVA was determined viable (parametric). P-values below 0.05 were considered statistically significant at a 95% confidence interval.

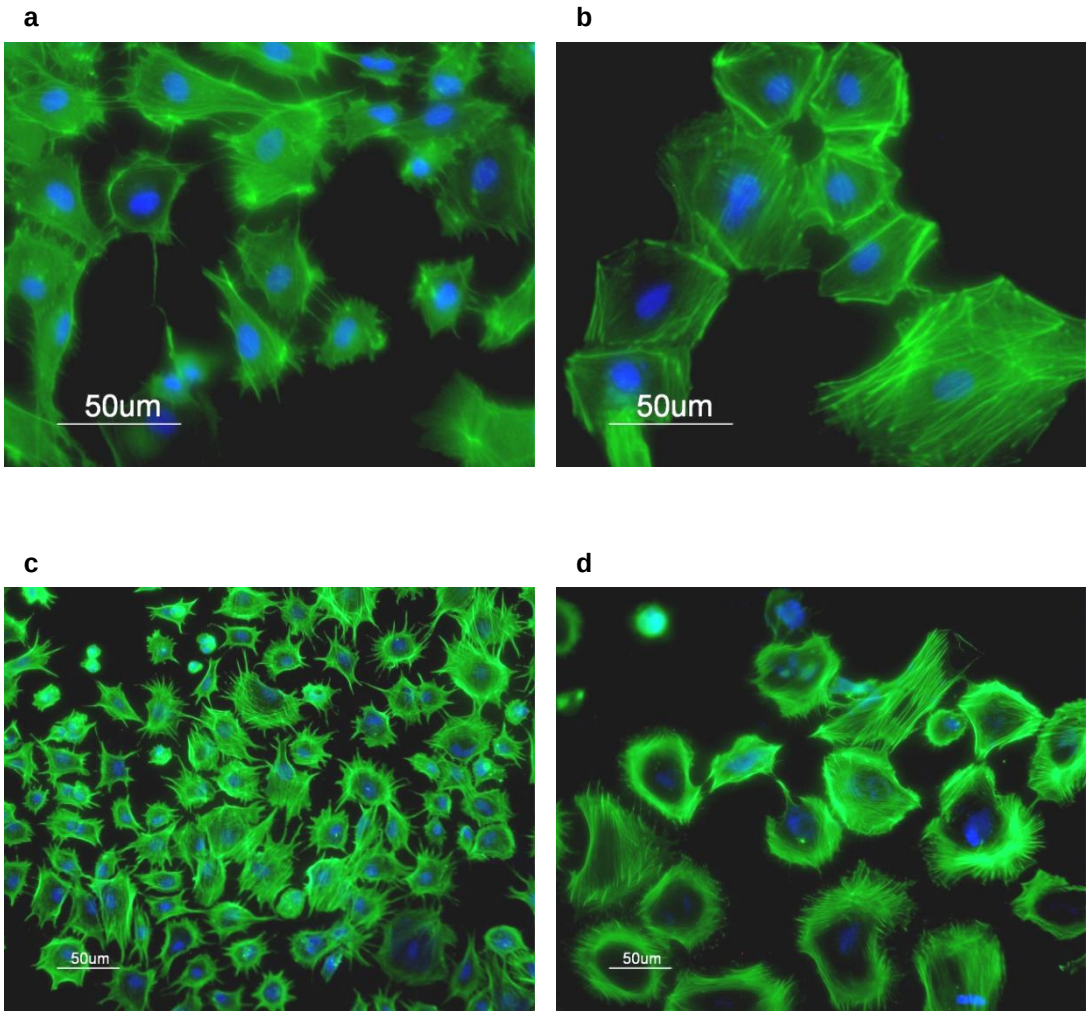
## 2.3. Results

### 2.3.1. Isolation of Dental Pulp Progenitors

Both of the human fibronectin adherent (hFNA) and human non-adherent (hNA) cell populations proliferated, formed colonies and were culture expanded with ease. The human $P75^{+}$ ($hP75^{+}$) cells did not proliferate after its isolation but the negative flow-through population ($hP75^{-}$) did proliferate. After more than four failed attempts to sort $hP75^{+}$ cells directly from the enzyme-dissociated pulp tissue, a successful $hP75^{+}$ population was obtained by culturing the dissociated heterogeneous pulp population in a T75 flask (Starstedt, Germany) for one passage before trypsining the cells (as in section 2.2.3) to create a cell suspension for P75 sorting (section 2.2.7). The rodent whole pulp cells, rodent FNA (rFNA), rodent non-adherent (rNA), rodent $P75^{+}$ ($rP75^{+}$) and rodent $P75^{-}$ ($rP75^{-}$) populations were proliferative and was cultured without difficulty.

### 2.3.2. Progenitor Cell Morphology

rFNA, rNA, $rP75^{+}$ and $rP75^{-}$ clonal cells were cultured on glass cover-slips to allow them to be stained with phalloidin-FITC to determine morphological differences. The rFNA cells were shown to have multiple cellular projections and were similar in morphology to fibroblasts (figure 2.1a) and its rNA counterpart was similar in size to the rFNA, although oblong-like with no apparent projections (figure 2.1b). The $rP75^{+}$ cells were mainly smaller (~25 μm diameter), approximately half the size of the rFNA/rNA (~50 μm diameter) with more projections (stellate) compared with the rFNA population (figure 2.1c). The negatively selected $rP75^{-}$ were larger cells than the $rP75^{+}$ (and similar size to the rFNA/rNA) but with more varying cell




**<u>Figure 2.1:</u> Actin cytoskeleton staining of rFNA (a) compared with rNA (b) cells (both ×20 magnification), and $rP75^{+}$ (c) compared with $rP75^{-}$ (d) cells (both ×10 magnification). Green fluorescence is Phalloidin-FITC staining, and blue fluorescence is the nuclei bisBenzimide counter staining.**

morphologies, some were rounded and some were similar in morphology to fibroblasts and had variable degrees of cellular extrusions (figure 2.1d).

Time-lapse imaging of a hFNA clonal population over 1-3 days and continued to 3-5 days (supplementary CD video1 and video2 respectively) demonstrated cell proliferation with the cells in a repeated cycle of becoming fibroblast-like in morphology, dividing into more rounded cells then forming fibroblast-like cells again. As the cell culture became confluent and densely populated, the cell numbers continued to expand and formed a new layer of cells above the confluent cell layer.

### 2.3.3. Comparing Rodent DPSC Colony Forming Efficiency

Comparing rFNA to the corresponding rNA CFEs (figure 2.2) showed a highly significant difference ($P < 0.01$) at day 3 with the rFNA having higher CFEs at 0.003 ±0.001 compared to the rNA with 0.002 ±0.001. There was a reduced but still significant difference ($P < 0.05$) of CFEs at day 6 of culture, where the rFNA CFE was 0.02 ±0.004, twice the amount of the rNA with 0.01 ±0.002.

Day 10 and day 12 of culture demonstrated an increase in CFEs but with no significant differences ($P > 0.05$) between the rFNA and rNA CFEs. However, when considering the large SEM values, it appears the rFNA had a higher CFE compared with the rNA where at day 10 the rFNA had 0.07 ±0.02 compared with the rNA at 0.04 ±0.01 and at day 12 the rFNA had 0.08 ±0.02 compared with the rNA at 0.04 ±0.01.

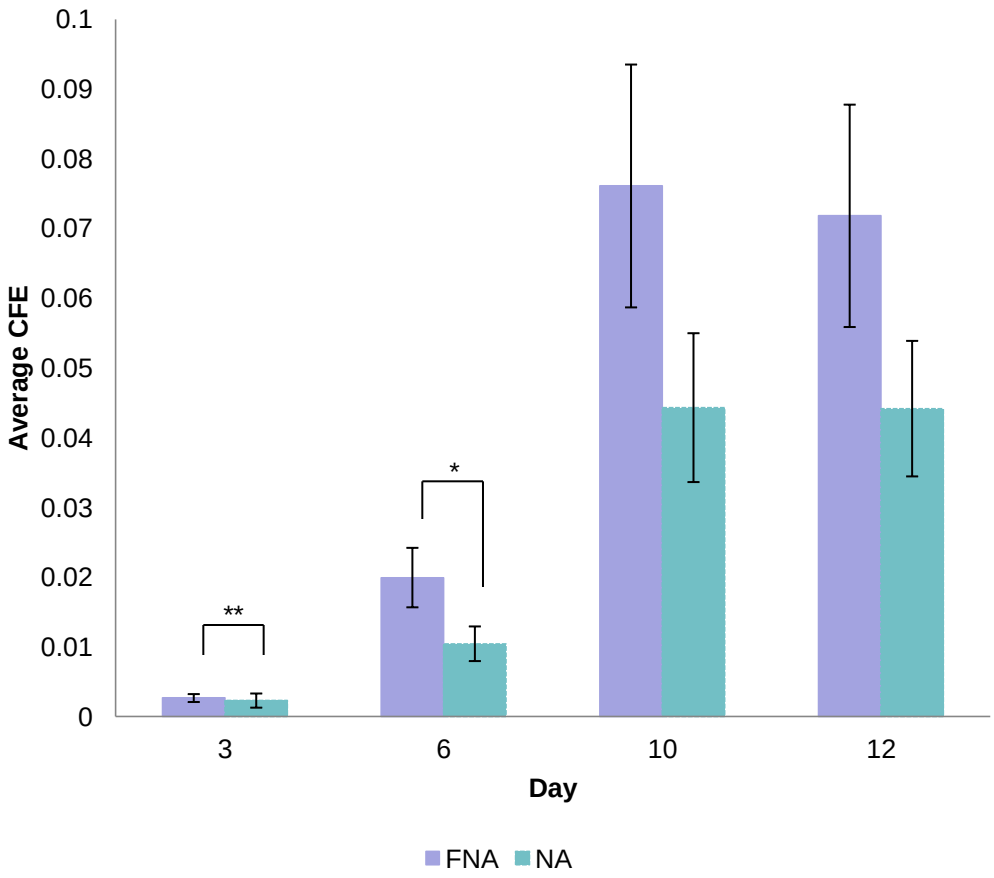



**Figure 2.2:** **Comparison of CFEs between rFNA and rNA clonal populations. There is a highly significant difference in CFE at day 3 and a significant difference on day 6, but no significant differences on day 10 and 12. Error bars illustrates SEM.**

**rFNA and rNA: n = 30**
*** P <0.05, ** P <0.01**

For the rodent P75 populations (figure 2.3) no colonies were apparent on day 3 for the $rP75^{-}$, so statistical comparison was not made with the $rP75^{+}$ (CFE of $6 \times 10^{-5} \pm 4 \times 10^{-5}$). On day 6 the $rP75^{+}$ showed an increase of CFE at $0.032 \pm 0.009$ and the $rP75^{-}$ CFE at $0.009 \pm 0.003$ where neither had statistically significant CFE increases. With 10 days of culture the $rP75^{+}$ had a CFE of $0.07 \pm 0.02$, over three times higher CFE and significantly more than the $rP75^{-}$ at $0.02 \pm 0.006$. After 12 days there was still a significant difference between the two with the CFE of the $rP75^{+}$ remaining three times as high at $0.13 \pm 0.02$ whereas the $rP75^{-}$ was at $0.04 \pm 0.01$.

### 2.3.4. Comparing Human DPSC Colony Forming Efficiency

For human dental pulp progenitors, CFE was only determined using the fibronectin preferentially selected cells. The $hP75^{+}$ selection process initially had difficulties in harvesting cells that proliferated into colonies for determining the CFE. There were generally higher CFE with the hFNA compared with the hNA populations (figure 2.4). However, the large SEM values and the statistical testing indicated that there were no significant difference between the hFNA and the hNA populations. At day 3 of culture no colonies were formed by the hFNA or the hNA populations, but by day 6 only the hFNA had formed colonies with CFE of $6.5 \times 10^{-5} \pm 6.5 \times 10^{-5}$. With 10 days of culture the CFE of hFNA was $0.005 \pm 0.002$ and the hNA at $0.002 \pm 0.001$. On day 12 the CFEs were $0.007 \pm 0.003$ for the hFNA and slightly less at $0.006 \pm 0.002$ for the hNA.

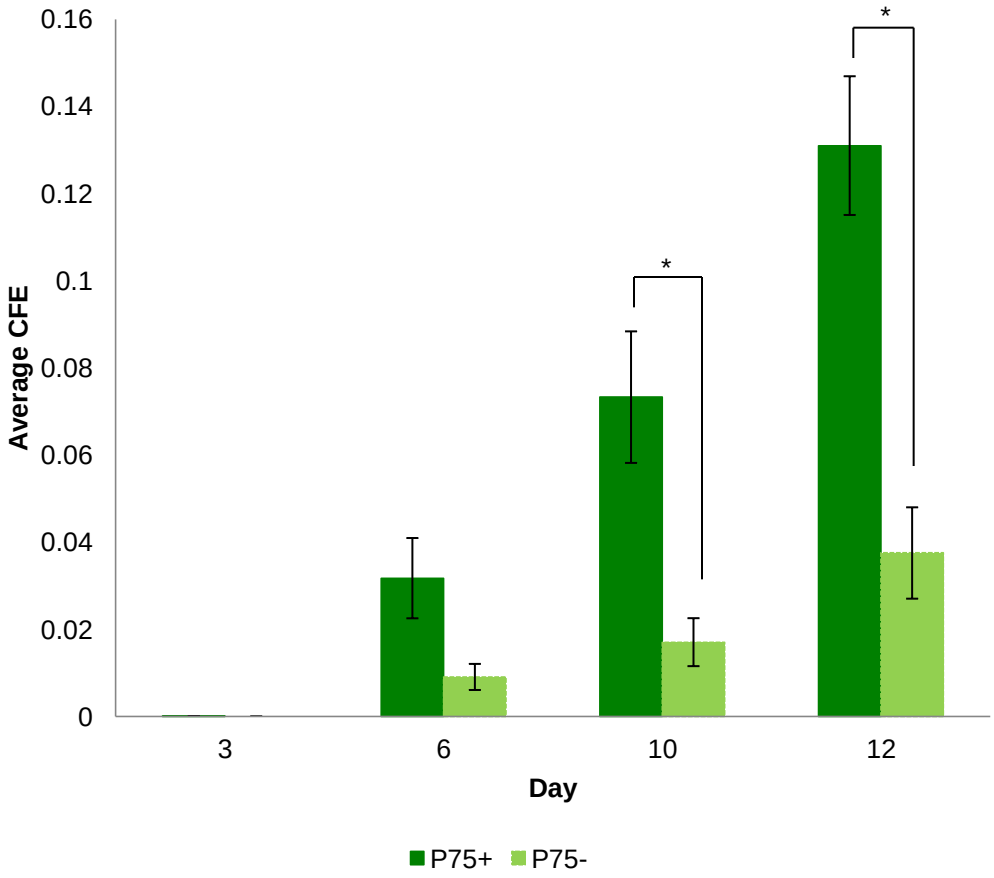
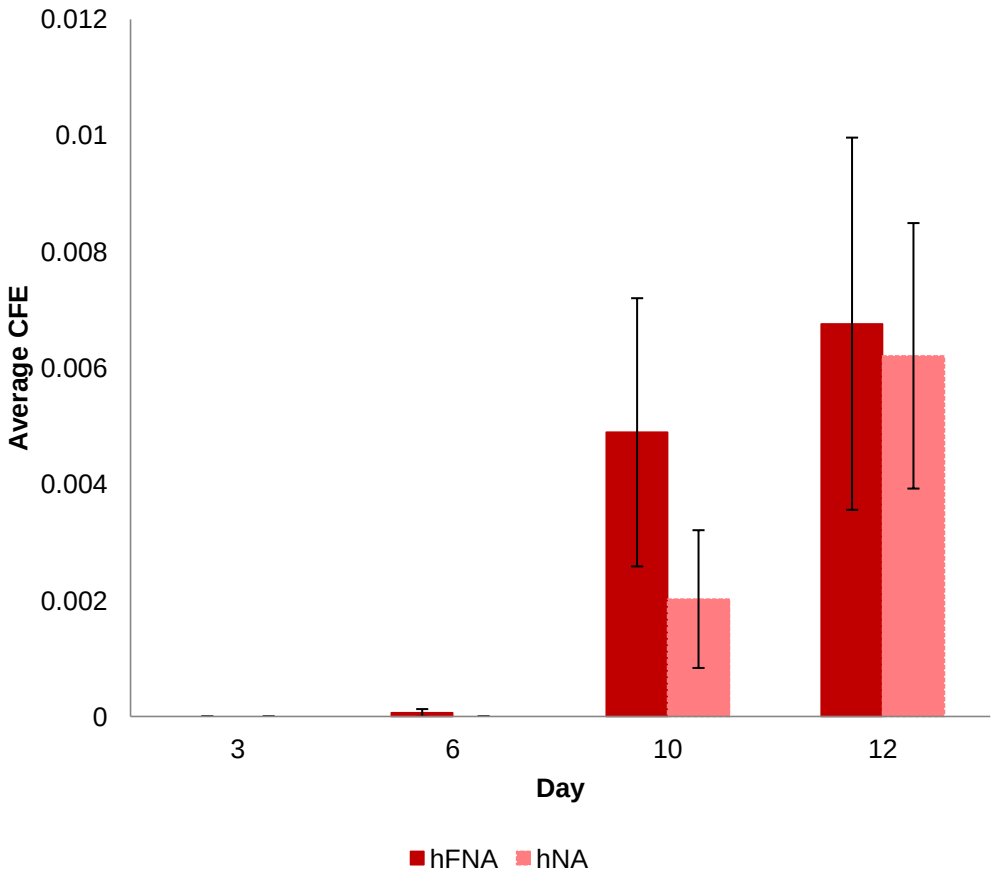



**Figure 2.3: Comparison of CFEs of rP75$^{+}$ and rP75$^{-}$ populations. Significant difference in CFE between positive and negative populations were apparent from day 10 onwards. The error bars represent the SEM.**

**P75$^{+}$ n = 6, P75$^{-}$ n = 3**
*** p<0.05**




**<u>Figure 2.4:</u> The CFEs between hFNA and hNA populations. The results indicate an increase of CFE with culturing, where comparison between hFNA and hNA showed no significant differences. The SEM is shown by the error bars.**

**hFNA and hNA: n=10**

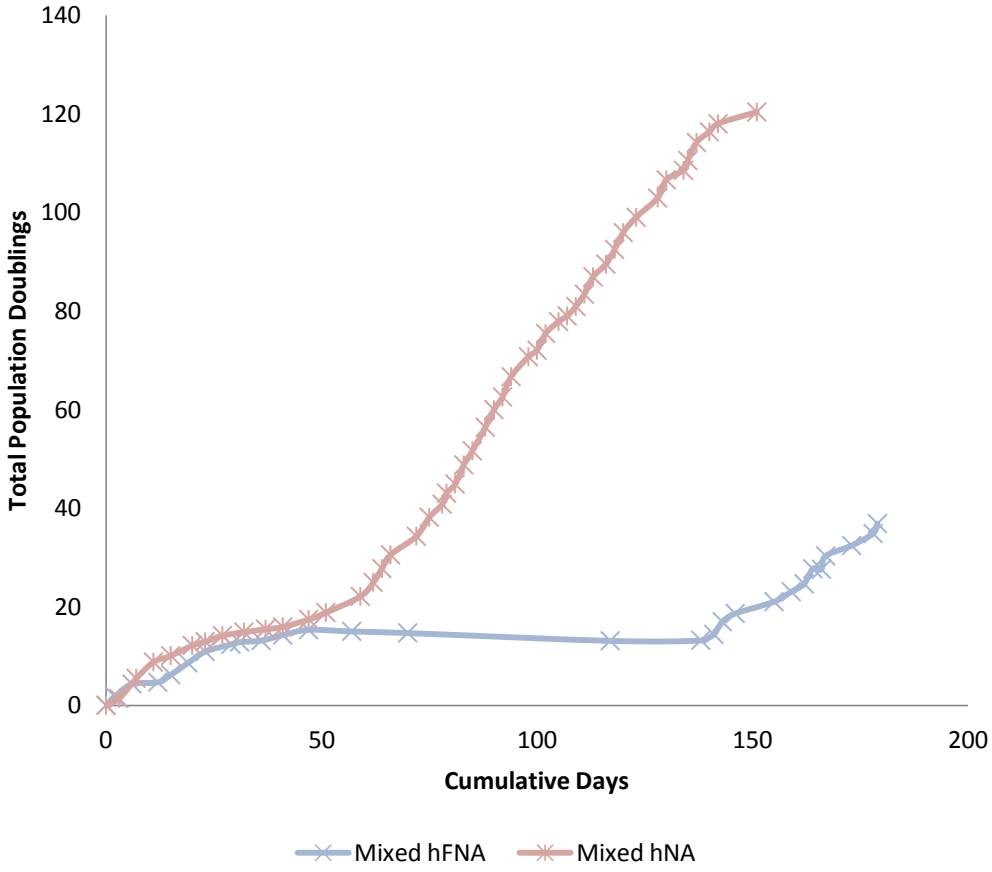
### 2.3.5. The Growth Kinetics of Dental Pulp Isolated Progenitor Cells

#### 2.3.5.1. Human DPSC Population Doublings

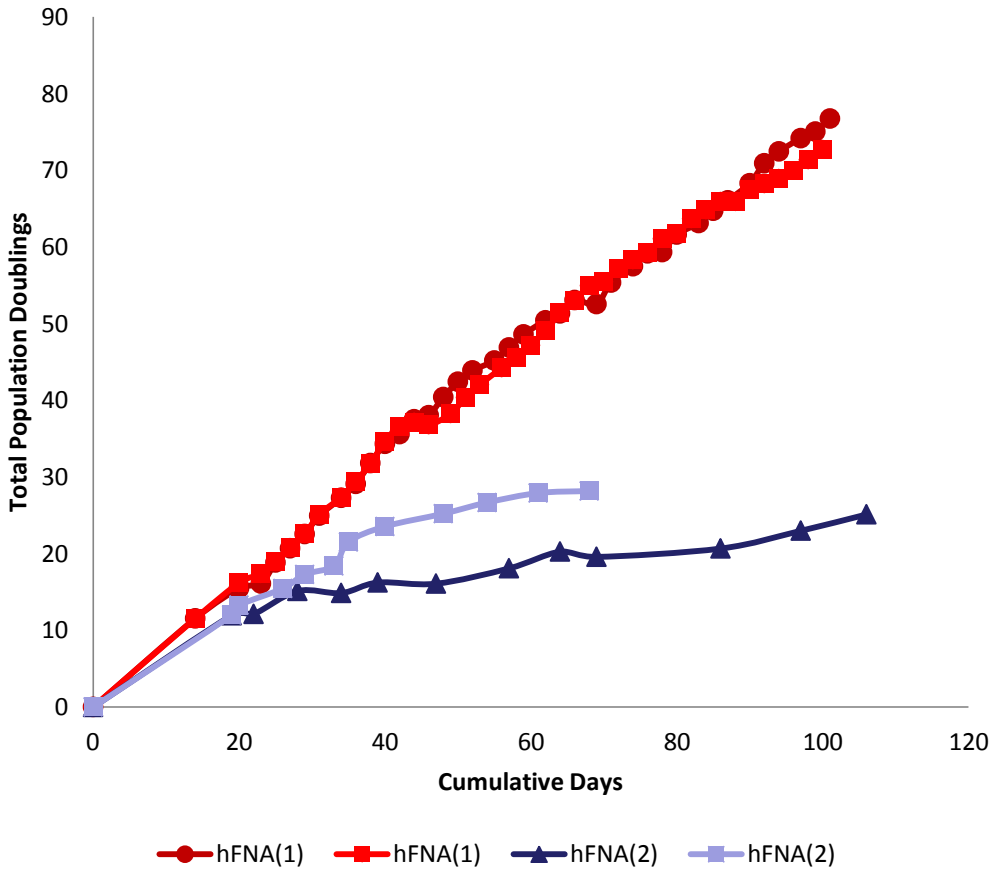
The population doublings (PDs) of dental pulp progenitors taken from the same patient were qualitatively compared. When the colonies were allowed to merge and form a heterogeneous fibronectin adherent population (mixed hFNA) there was an initial slow increase in PDs (a lag in growth) that reached 15 PDs after 50 days of culture (figure 2.5). The PDs then plateaued before showing signs of increase after another 90 days of culture (140 days in total), reaching 37 PDs by 180 days of culture. The heterogeneous non-adherent population (mixed hNA) had similar PDs to the mixed hFNA until day 50 of culture where they then rapidly increased in PDs reaching around 72 PDs at day 100 and continued increasing to 120 PDs by 150 days of culture.

The comparison of clonal populations of hFNA from the same patient (figure 2.6) revealed a similar PD pattern, however the different patient clones yielded different rates of doubling. Clones from one patient had reached over 70 PDs after 100 days of culture whilst a different patient clone reached over 20 PDs over the same period. The PDs were compared between the hFNA and the hNA clonal populations from the same patient where similar growth kinetics were observed. However, the hNA clones initially had lower PDs than the hFNA clones but increased at a similar rate to the hFNA from day 100 onwards (figure 2.7).

Then the P75 cells sorted by MACS were also compared (figure 2.8) which showed the $hP75^{+}$ populations had seemingly higher PDs compared with the $hP75^{-}$ (observed




**<u>Figure 2.5:</u> The heterogeneous populations, mixed hFNA and mixed hNA had similar PDs when cultured initially. The PDs diverged after 50 days with mixed hNA increase in PDs and the mixed hNA plateauing before it increased after 140 days. Each data-point indicates day of cell counts to calculate PDs.**




**<u>Figure 2.6:</u> The PDs of 4 hFNA clones from two patients (1 or 2). Results shows 2 distinct groups of PDs, hFNA(1) having more rapid increase in PDs, compared with slower hFNA(2). Each data-point indicates day of cell passaging and cell counts to calculate PDs.**

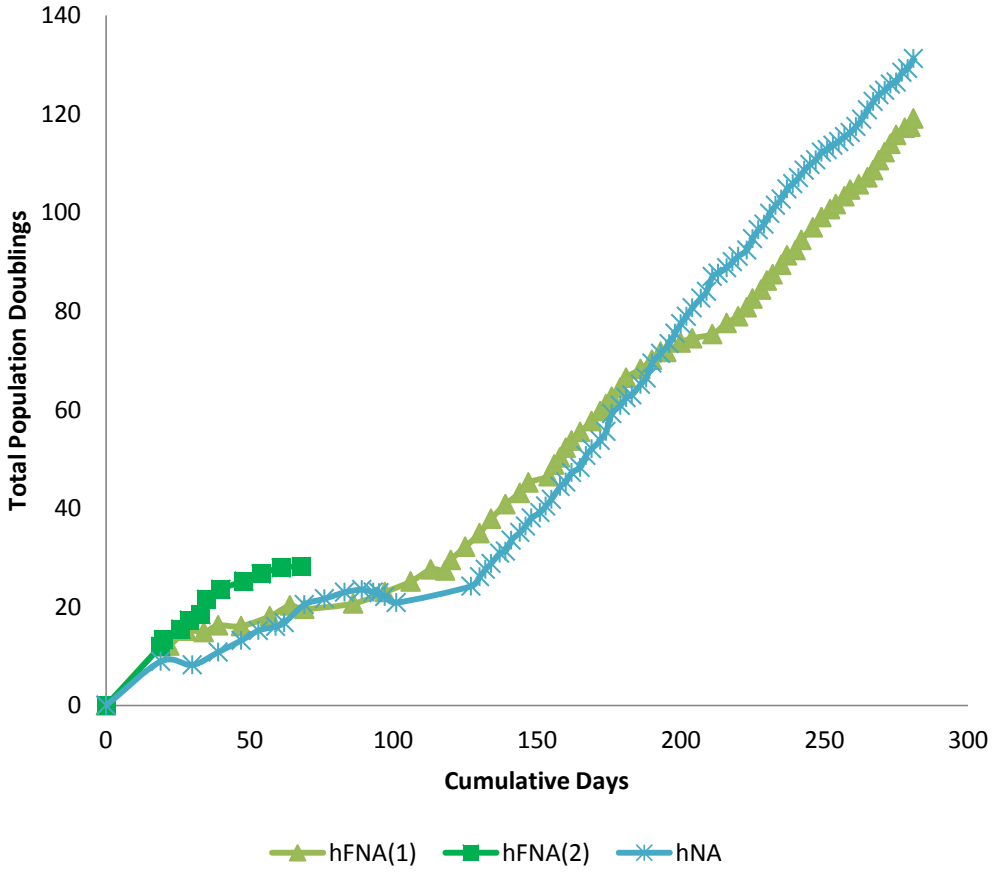



**Figure 2.7: The PDs of 2 hFNA clones and the corresponding patient-matched hNA clone. Results show hFNA clones initially have higher PDs, before the hNA increased and doubled at a similar rate. Each data-point indicates day of cell passaging and cell counts to calculate PDs.**

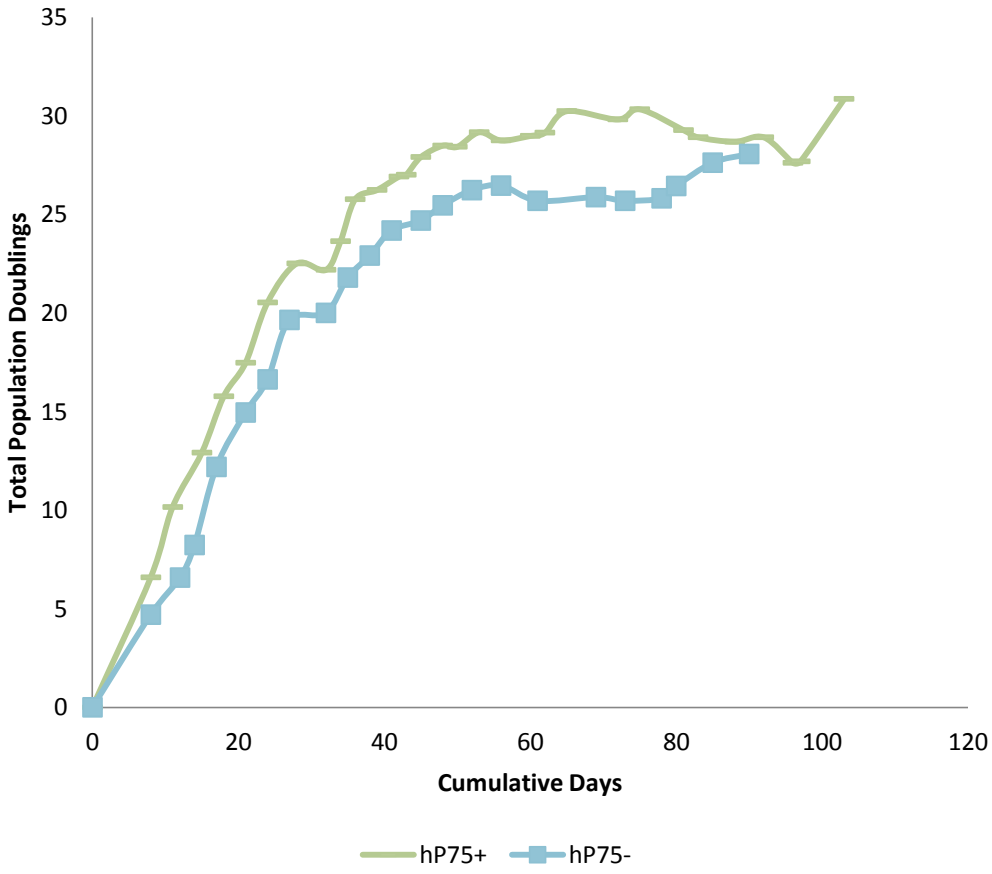



**Figure 2.8: The human PDs of hP75$^{+}$ and hP75$^{-}$ populations showed a higher PD with hP75$^{+}$ compared with P75$^{-}$, although both plateaued after 40 days of culture. Each data-point indicates day of passaging and cell counts to calculate PDs**

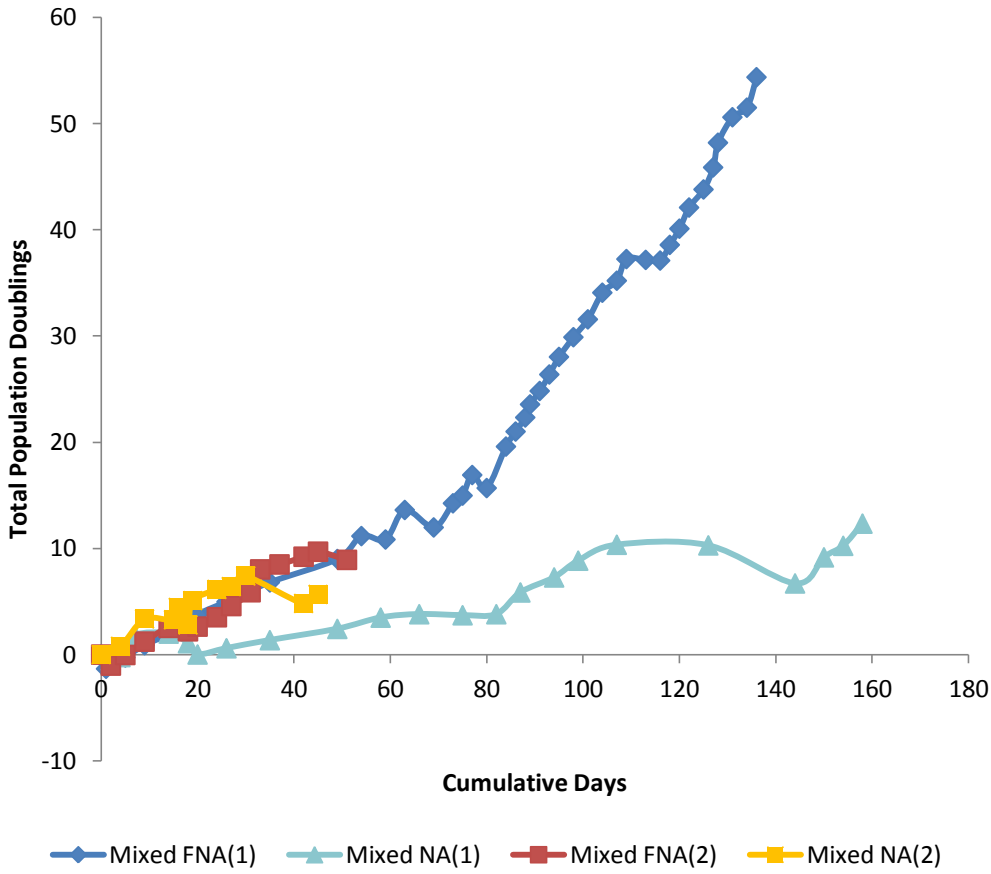
from just one population of both) although both had similarly plateaued in PDs following 40 days of culture.

#### 2.3.5.2. Rodent DPSC Population Doublings

Fibronectin preferentially selected rodent cell colonies were allowed to merge and form heterogeneous populations that were either fibronectin adherent (mixed rFNA) or non-adherent (mixed rNA). Qualitative comparison of the mixed rFNA and the corresponding mixed rNA populaions from the same experiments (experiment "1" and "2") revealed different growth kinetics (figure 2.9). Both populations displayed a lag in growth up to 50-60 days of culture before increasing in PDs. The mixed rFNA population had higher PDs compared with the mixed rNA particularly when cultured for a longer period. This was established when the mixed rFNA(1) reached 30 PDs and the mixed rNA(1) only reached 9 PDs after 100 days of culture.

With the comparison of homogeneous populations the PDs of the rNA were generally less than the rFNA clones. The rFNA and rNA clones all demonstrated a similar growth-lag-growth pattern, one of the rFNA clones increased and plateaued on day 13 of culture before it increased again after 90 days and reached 94 PDs after 200 days of culture. The two rNA clones increased in doublings between at 140 and 180 days respectively (figure 2.10).

The selection of multiple $rP75^{+}$ clones from the same experiment revealed similar growth kinetics to each other. Each of the $rP75^{+}$ clones was capable of producing high PDs. Following an initial lag of growth there was a rapid increase of PDs from day 100 of culture before finally reaching 60 PDs by day 200 (figure 2.11).




**<u>Figure 2.9:</u> The PDs of heterogeneous mixed rFNA and it's corresponding mixed rNA from the same experiment (1 or 2). Results indicated similar growth kinetic between the mixed FNA and mixed NA populations, reaching plateau before increasing again. The mixed rFNA generally had a higher PD compared with the mixed rNA. Each data-point indicates day of cell counts to calculate PDs.**

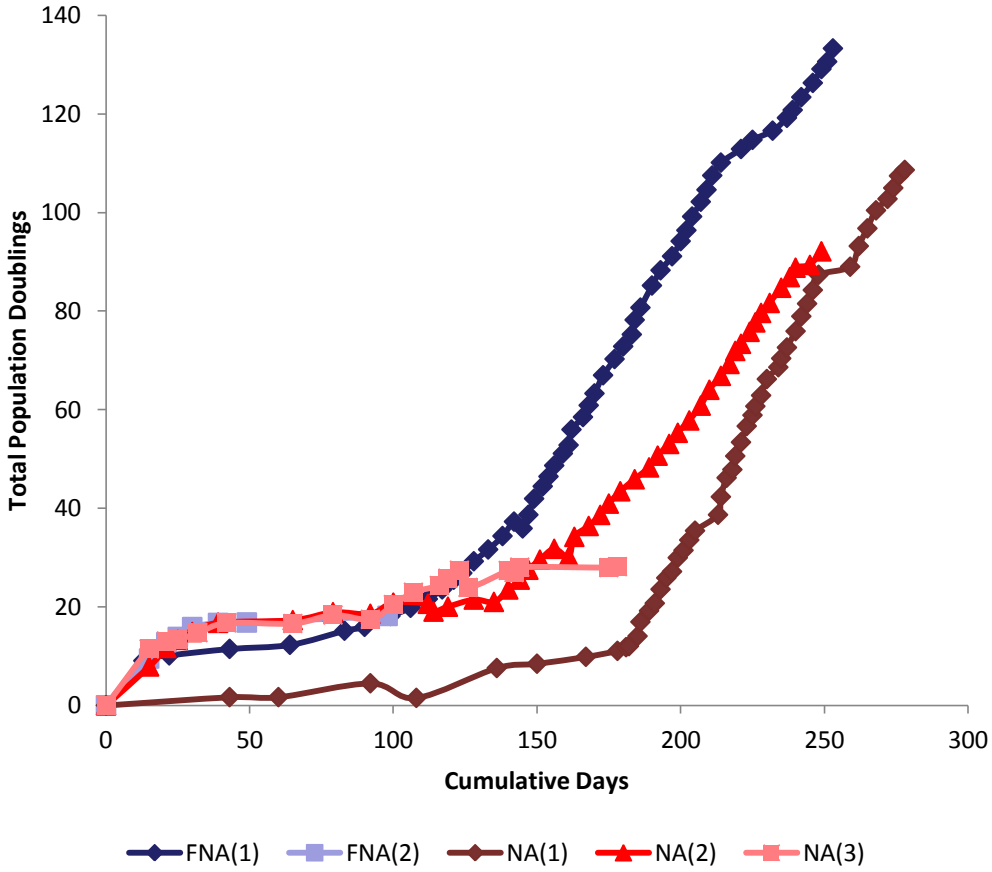



**Figure 2.10:** **The PDs of 2 rFNA clones and 3 rNA clones from the same experiment. Results showed the distinct increase-lag-increase of PDs. The two rFNA clones shared similar initial PDs, the rNA clones had lower PDs than rFNA clones. Each data-point indicates day of cell counts to calculate PDs.**

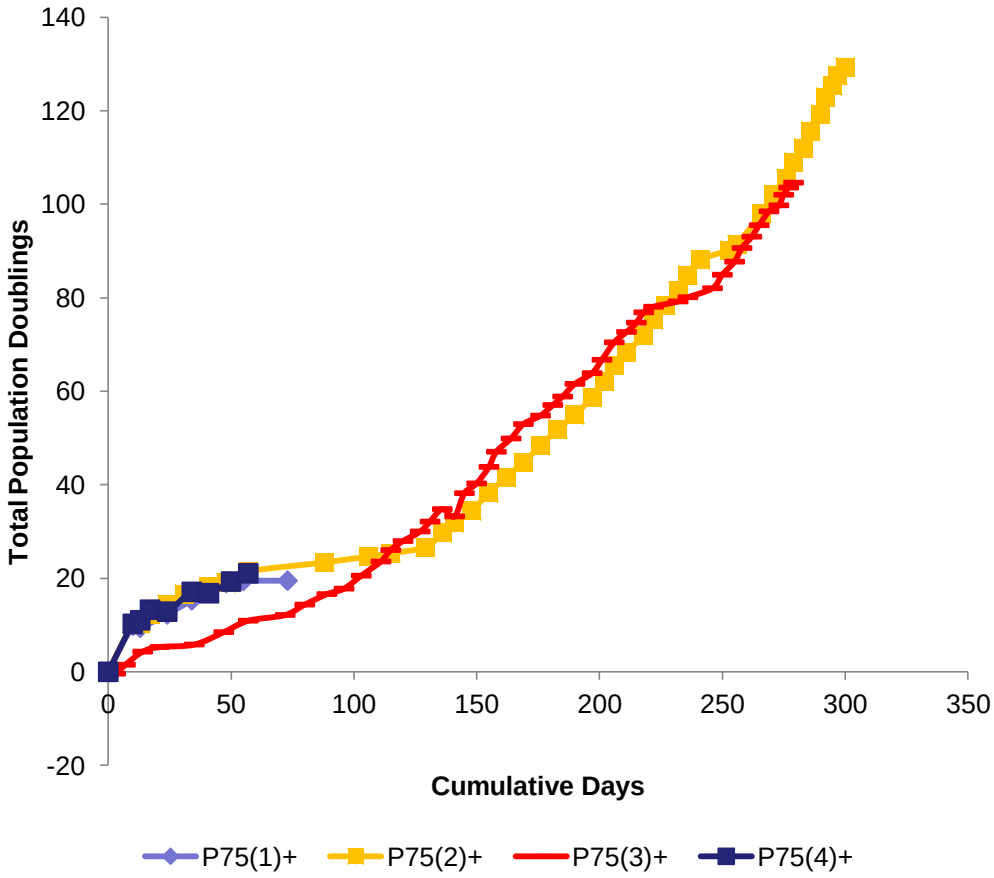



**Figure 2.11: The PDs of 4 rP75$^{+}$ from the same experiment. Results show the clones had similar PD kinetics, particularly with long term culture Each data-point indicates day of cell counts to calculate PDs.**

### 2.3.6. Gene Marker Expression in Human DPSCs

A summary of multipotent and pluripotent marker expression in human dental pulp tissue and its isolated cell populations can be found in table 2.3.

#### 2.3.6.1. Mesenchymal Markers of Human DPSCs

A triplicate of markers chosen as typical MSC markers included CD105 (*eng*, or endoglin), CD146 (*mcam*, or melanoma cell adhesion molecule) and MSX-1 (*msx1*, or Msh homeobox-1). These markers were expressed in whole pulp tissue immediately after fibronectin selection and up to early culture (hFNA at 13 PDs, hNA at 15 PDs) as representations of heterogeneous cell populations (figure 2.12a). Some of these markers were lost by later PD (hFNA at 36 PDs, hNA at 120 PDs).

Using fibronectin selection and clonal expansion to produce a homogenous population, the hFNA and hNA clones expressed mesenchymal markers during early culture (figure 2.12b). However, as with the heterogeneous populations the markers were mostly absent by 91 PDs (220 days) as shown by the hNA clone and 106 PDs (126 days) for the hFNA clone (around 50 passages for both). With P75 sorting of dental pulp cells into $hP75^{+}$ and $hP75^{-}$ populations, both populations expressed mesenchymal markers during early culture (23 and 21 PDs respectively). Only the $hP75^{+}$ population maintained all marker expression at later culture (30 PDs), whilst $hP75^{-}$ (at 32 PDs) was $CD146^{+}$, but $CD105^{-}$ and $MSX\text{-}1^{-}$ (figure 2.12c). Positive controls 501mel and H9 both positively expressed the mesenchymal markers whilst the water and RT-negative experimental controls had no expression (figure 2.12d).

**Table 2.3:** Summary of the multipotent and pluripotent markers expressed in: human dental pulp, heterogenic hFNA/hNA, clonogenic hFNA/hNA and $hP75^{+}/hP75^{-}$ populations at early and late cultures. The + represents positive expression and – represents absence of expression.

| *Multipotent and Pluripotent Markers* | | *Human Dental Pulp* | *Early Culture Hetero hFNA / hNA* | *Late Culture Hetero hFNA / hNA* | *Early Culture Clonal hFNA / hNA* | *Late Culture Clonal hFNA / hNA* | *Early Culture hP75+ / hP75-* | *Late Culture hP75+ / hP75-* |
|---|---|---|---|---|---|---|---|---|
| Mesenchymal | CD105 | + | + / + | – / – | + / + | –/ + | + / + | + / – |
| | CD146 | + | + / + | + / + | + / + | – / – | + / + | + / + |
| | MSX-1 | + | + / + | – / – | + / + | – / – | + / + | + / – |
| Neural Crest | P75 | + | + / + | + / – | + / + | – / – | + / + | + / – |
| | Slug | + | + / + | + / + | + / + | – / – | + / + | + / – |
| | Snail | + | + / + | + / – | + / + | – / – | + / + | + / – |
| | Twist | + | – / – | – / – | + / + | – / – | + / + | + / + |
| Embryonic | Oct4a | + | + / + | + / + | + / + | – / – | – / – | – / + |
| | Nanog | + | + / + | – / – | + / + | – / + | – / – | – / – |
| | hTERT | – | – / – | – / – | – / – | – / – | – / – | – / – |
| | hTR | + | + / + | + / + | + / + | – / – | + / – | – / + |
| Developmental Notch Receptors | Notch1 | + | + / + | + / + | + / + | + / – | + / + | + / + |
| | Notch2 | + | + / + | – / – | + / + | – / + | + / + | + / + |
| | Notch3 | + | + / + | + / + | + / + | + / + | + / + | + / + |
| Developmental Notch Ligands | Jagged1 | + | + / + | + / + | + / + | + / + | + / + | + / + |
| | Jagged2 | + | + / + | + / + | + / + | + / + | + / + | + / + |

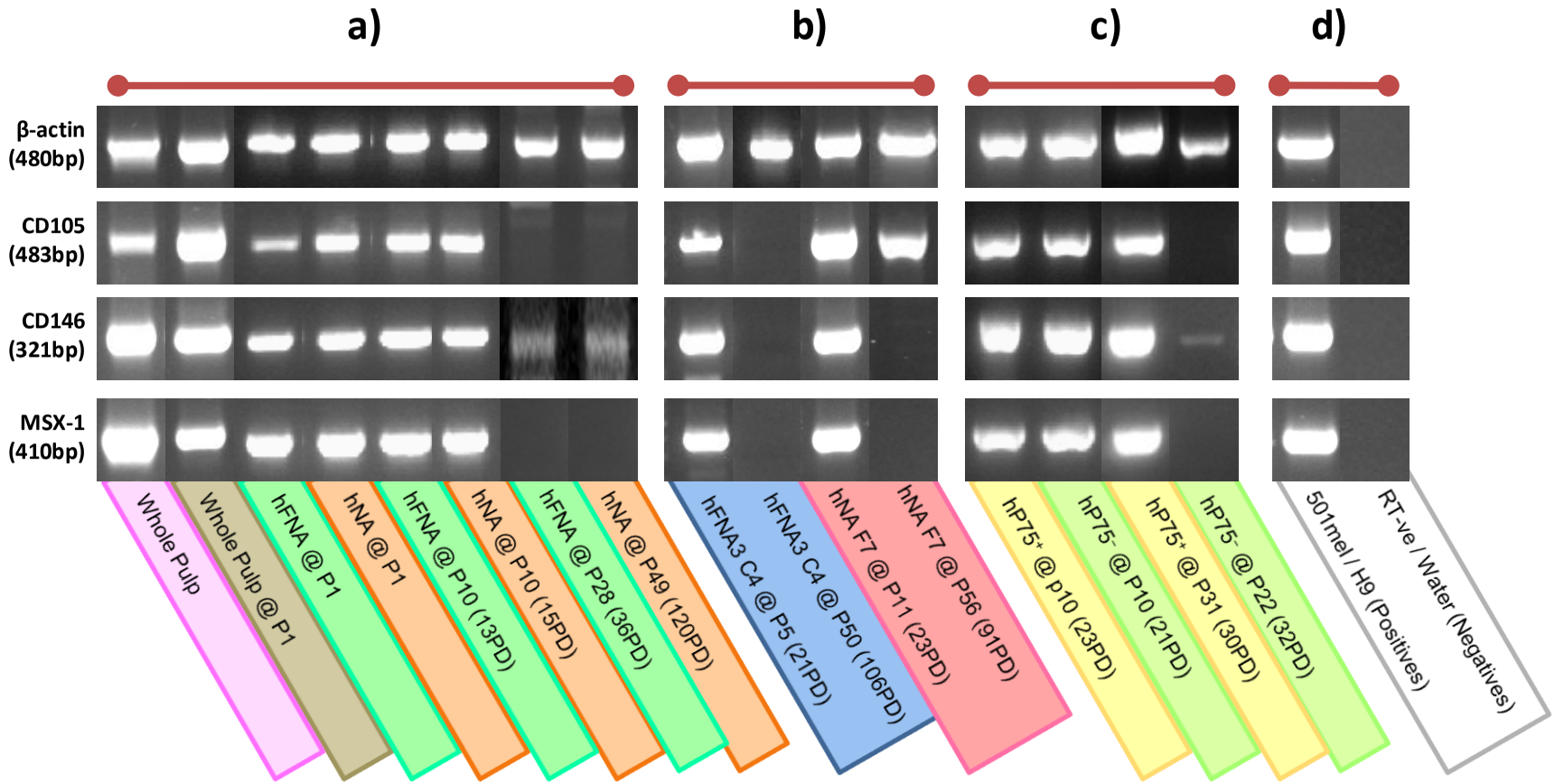



**Figure 2.12:** Mesenchymal markers expressed in human dental pulp heterogeneous populations (a), present in fibronectin selected early culture (b), hP75 sorted (c) and positive control (d) samples.

### 2.3.6.2. Neural Crest Markers of Human DPSCs

Markers for neural crest cells were also investigated using four common neural crest markers P75 (*ngfr*), Slug (*snai2*), Snail (*snai1*) and Twist (*twist1*). The heterogeneous dental pulp populations (figure 2.13a) illustrated how the whole pulp population of cells expresses all the neural crest markers. Once the dental pulp cells were fibronectin sorted and the colonies were allowed to merge, both the hFNA and the hNA lost Twist expression. However, the remaining neural crest markers were mostly maintained into later PD. The hFNA at 36 PDs maintained $P75^{+}$, $Slug^{+}$ and $Snail^{+}$ expression but the hNA at 120 PDs had only $Slug^{+}$ expression.

With the fibronectin selection for homogeneous clonal populations, early culture of both hFNA (21 PDs) and hNA (23 PDs) clones expressed all neural crest markers, this included Twist which was absent in the heterogeneous selected populations (figure 2.13b). These neural crest markers were not maintained and were absent at 106 and 91 PDs for the hFNA and hNA clones respectively.

When the dental pulp was sorted into $hP75^{+}$ and $hP75^{-}$ populations both expressed all the neural crest markers at early PD (23 PDs and 21 PDs respectively). With later PDs, the $hP75^{+}$ (30 PDs) maintained all the neural crest markers whilst the $hP75^{-}$ (32 PDs) had only $Twist^{+}$ present with the remaining markers absent (figure 2.13c). Positive controls 501mel and H9 had both positively expressed the neural crest markers and the water and RT-negative experimental controls had no expression (figure 2.13d).

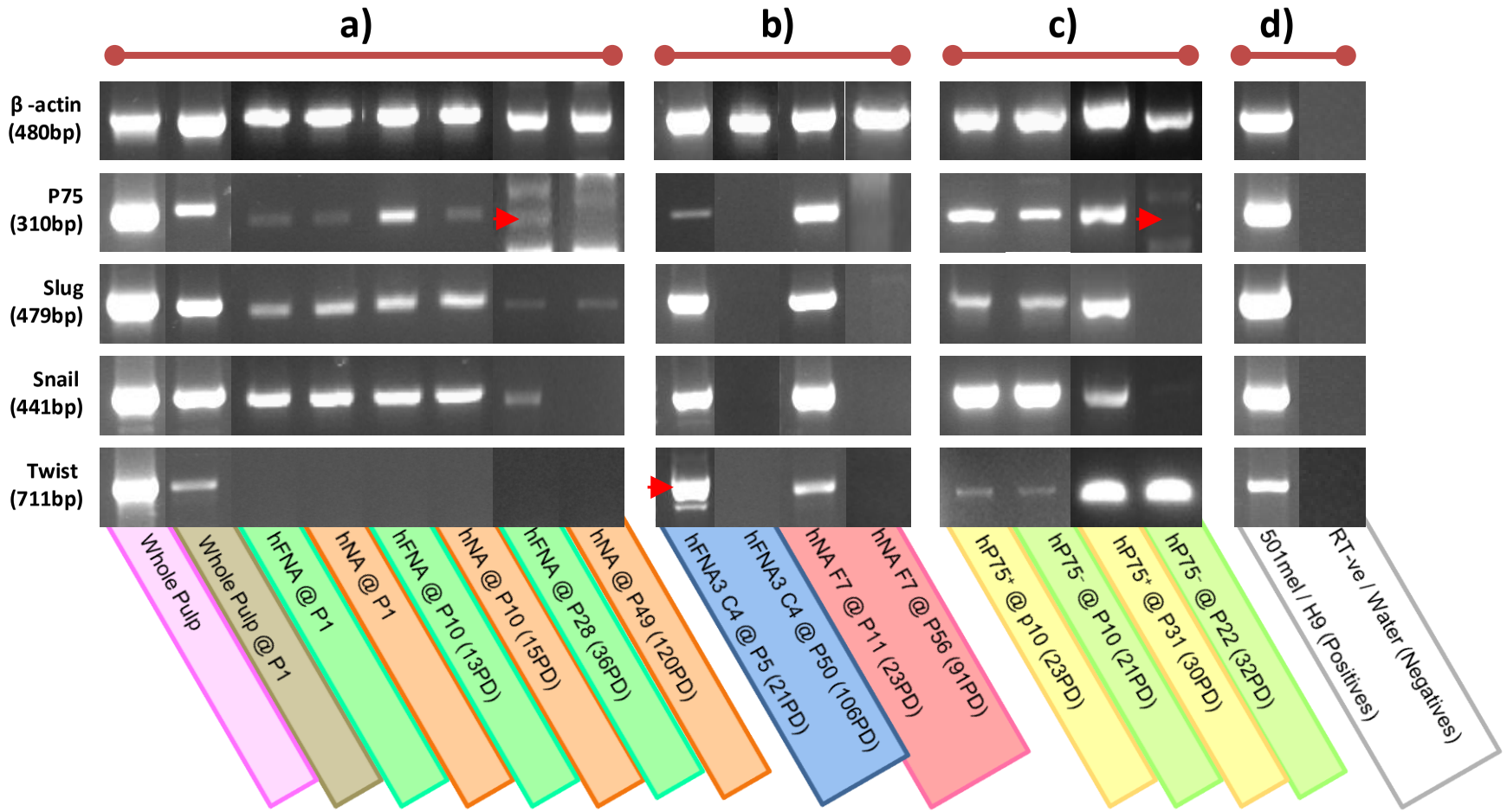



**Figure 2.13:** Neural crest markers were mostly expressed in human whole pulp and heterogeneous populations (a), early cultured fibronectin selected clones (b), hP75 sorted cells (c) and in the positive control (d) samples. Arrowheads indicate correct band location.

### 2.3.6.3. Embryonic Markers of Human DPSCs

Embryonic markers Oct4a (*pou5f1*), Nanog, hTERT (telomerase) and hTR (telomerase RNA component or TERC) were examined in human dental pulp progenitors. The heterogeneous pulp populations were $Oct4a^{+}$, $Nanog^{+}$ and $hTR^{+}$ but were $hTERT^{-}$ (figure 2.14a). With fibronectin selection the later PDs (hFNA 36 PDs and hNA 120 PDs) revealed most of these markers were expressed (Oct4a and hTR) except for Nanog which was absent in these heterogeneous cultures. A similar expression pattern was shown with the fibronectin isolated homogenous populations, the hFNA and hNA clones were $Oct4a^{+}$, $Nanog^{+}$ and $hTR^{+}$ during 21 and 23 PDs respectively (figure 2.14b). These markers were mostly absent by later culture where hFNA (106 PDs) had no embryonic marker expression and the hNA (91 PDs) had $Nanog^{+}$ expression.

As like the heterogeneous samples hTERT was not expressed in both clonal populations which was also consistently same for the P75 sorted cell populations (figure 2.14c). The $hP75^{+}$ population was only $hTR^{+}$ during early 23 PDs but was negative for the other embryonic markers. At later PDs of $hP75^{+}$ (32 PDs) none of the embryonic markers were present. However, the $hP75^{-}$ population was positive for three embryonic markers $Oct4a^{+}$, $Nanog^{+}$ and $hTR^{+}$ at early 21 PDs but only $Oct4a^{+}$ and $hTR^{+}$ were maintained by 32 PDs. Positive controls 501mel and H9 both expressed the embryonic markers whilst the water and RT-negative experimental controls had no expression (figure 2.14d).

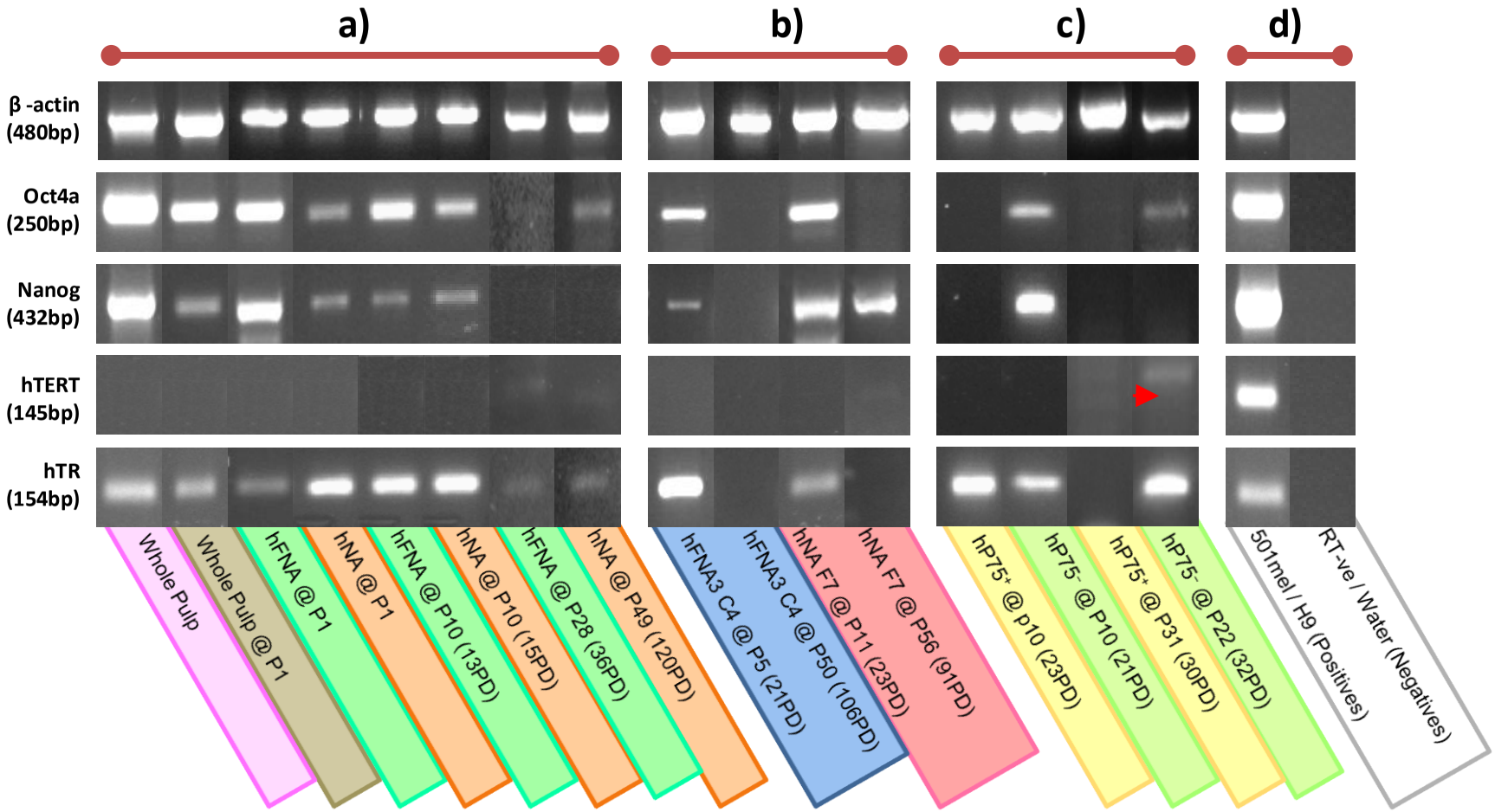



**Figure 2.14:** Embryonic markers expressed in human dental pulp heterogeneous (a), early cultured fibronectin selected clones (b), hP75⁻ (c) and positive controls (d). hTERT was absent from all human experimental samples. Arrowhead indicate correct band location.

#### 2.3.6.4. Early Developmental Markers of Human Progenitor Cells

Notch receptors Notch1, 2 and 3, with its associated ligands Jagged1 and 2 represents the early developmental markers in the isolated human dental pulp populations. The developmental markers were present in the heterogeneous populations even after fibronectin selected culture (figure 2.15a) although Notch2 was absent at later PDs (hFNA at 36 PDs and hNA at 120 PDs). Using fibronectin selected human dental pulp cells homogeneous clonal populations of the hFNA and hNA expressed the developmental markers at early culture with hFNA at 21 PDs and hNA at 23 PDs (figure 2.15b).

Longer culture saw the maintenance of nearly all markers, the hFNA clone was Notch2$^{-}$ by 106 PDs and hNA was Notch1$^{-}$ by 91 PDs. Alternatively, with the sorting of P75 cells by MACS, the hP75$^{+}$ and hP75$^{-}$ populations expressed all the developmental markers throughout its culture, maintaining expression up to 30 and 32 PDs respectively (figure 2.15c). Positive controls 501mel and H9 expressed the developmental markers and the water and RT-negative experimental controls had no expression (figure 2.15d).

### 2.3.7. Gene Marker Expression in Rodent DPSCs

A summary of multipotent and pluripotent marker expression in rodent dental pulp tissue and its isolated cell populations can be found in table 2.4.

#### 2.3.7.1. Mesenchymal Markers of Rodent DPSCs

Using the same band of mesenchymal markers as the human cell experiments (as in section 2.3.5, but designed for the rodent genome) the heterogeneous cell population

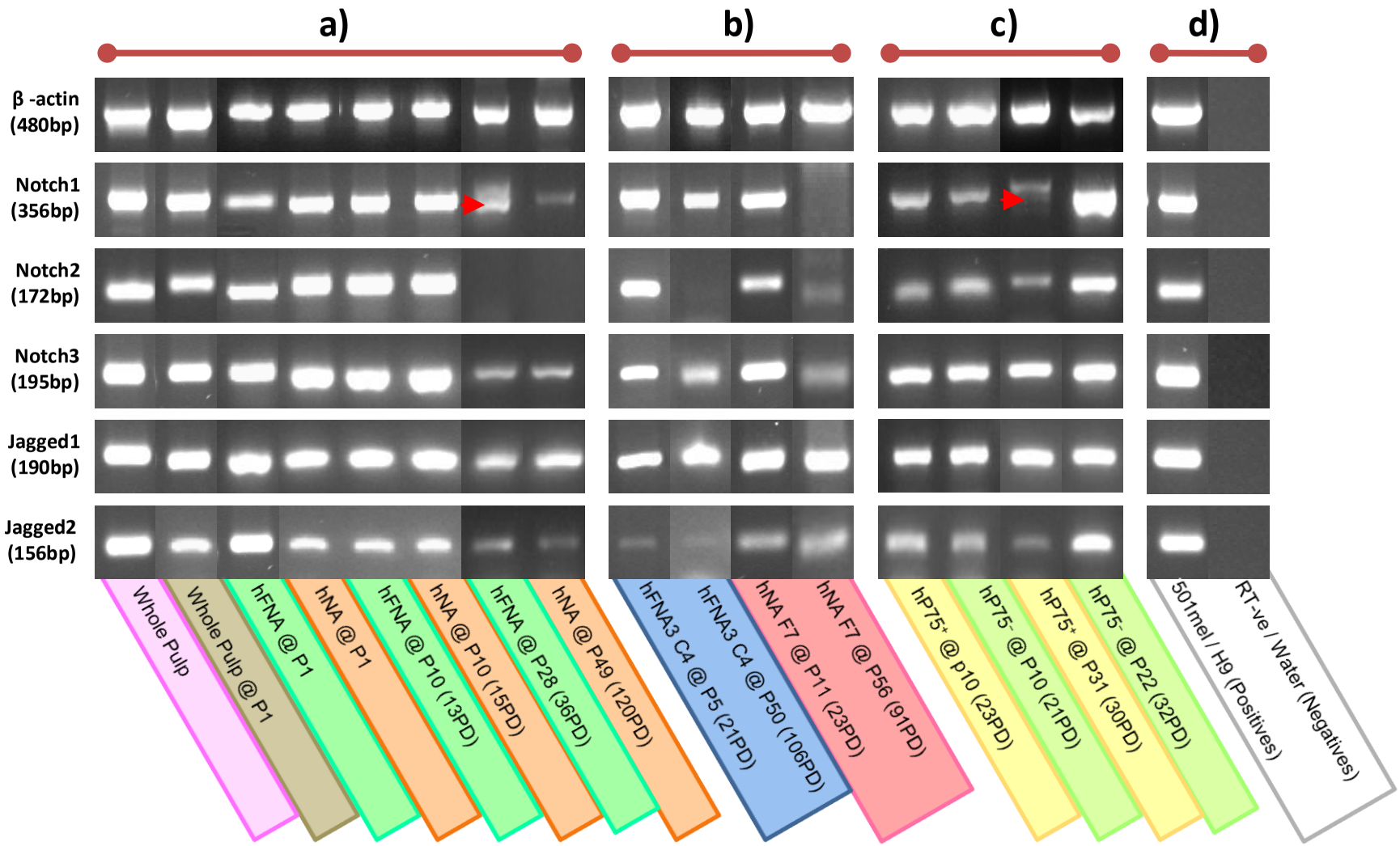



**Figure 2.15:** Developmental markers expressed in human pulp heterogeneous (a), fibronectin clonal (b) , hP75 sorted (c), and positive samples (d). Arrowheads indicate correct band location.

**Table 2.4:** Summary of the multipotent and pluripotent markers expressed in: rodent dental pulp, heterogenic rFNA/rNA, clonogenic rFNA/rNA and $rP75^{+}/rP75^{-}$ populations at early and late cultures. The + represents positive expression and – represents absence of expression.

| *Multipotent and Pluripotent Markers* | | *Rodent Dental Pulp* | *Early Culture Hetero rFNA / rNA* | *Late Culture Hetero rFNA / rNA* | *Early Culture Clonal rFNA / rNA* | *Late Culture Clonal rFNA / rNA* | *Early Culture rP75+* | *Late Culture rP75+* |
|---|---|---|---|---|---|---|---|---|
| Mesenchymal | CD105 | + | – / + | + / + | + / + | +/ + | + | + |
| | CD146 | + | + / + | + / + | + / + | + / – | + | + |
| | MSX-1 | + | + / + | + / + | + / + | + / – | + | + |
| Neural Crest | P75 | + | + / + | + / – | + / + | + / + | + | + |
| | Slug | + | + / + | + / + | + / + | + / + | + | + |
| | Snail | + | + / + | + / + | + / + | + / + | + | + |
| | Twist | – | – / – | + / + | + / + | + / – | + | + |
| Embryonic | Oct4 | – | – / – | – / – | – / – | – / – | – | – |
| | Nanog | – | – / – | – / – | – / – | – / – | – | – |
| | TERT | + | + / + | + / + | + / + | + / + | + | +? |
| | TR | + | + / + | + / + | + / + | + / + | + | + |
| Developmental Notch Receptors | Notch1 | + | + / + | – / + | + / + | + / + | + | + |
| | Notch2 | + | – / – | + / + | + / + | + / + | + | + |
| | Notch3 | + | + / + | + / – | + / + | + / + | + | + |
| Developmental Notch Ligands | Jagged1 | + | + / + | + / + | + / + | + / + | + | + |
| | Jagged2 | – | – / – | – / – | – / – | – / – | – | – |

of the rodent dental pulp expressed all the mesenchymal markers (figure 2.16a). With the heterogeneous fibronectin selected populations, where the cell colonies were allowed to merge, the rFNA initially was $CD105^{-}$, $CD146^{+}$ and $MSX\text{-}1^{+}$ at 1 PD before it was $CD105^{+}$, $CD146^{+}$ and $MSX\text{-}1^{+}$ after longer culture at 54 PDs. With the rNA heterogeneous population all the mesenchymal markers were absent at 2 PDs until at 12 PDs where all the mesenchymal markers were expressed again.

Alternatively, clonal cultures from fibronectin selected populations were cultured as homogenous populations. The rFNA clone at 23 PDs expressed all the mesenchymal markers (figure 2.16b) with faint $CD146^{+}$ by 119 PDs. The rNA clone was $CD105^{+}$, $MSX\text{-}1^{+}$ and $CD146^{+}$ at 11 PDs but only maintained $CD105^{+}$ expression by 108 PDs. Selection of $P75^{+}$ populations, clones C4 and C6 expressed all the mesenchymal markers and were maintained into 160 and 104 PDs respectively (figure 2.16c). The water and RT-negative experimental controls had no expression of mesenchymal markers (figure 2.16d).

### 2.3.7.2. Neural Crest Markers of Rodent DPSCs

The rodent dental pulp heterogeneous cell populations were investigated for the expression of neural crest markers. The whole pulp cell population are $P75^{+}$, $Slug^{+}$ and $Snail^{+}$, but $Twist^{-}$ (figure 2.17a). This was consistent even after fibronectin selection in both rFNA and rNA populations although at later culture the rFNA was found to be $P75^{+}$, $Slug^{+}$, $Snail^{+}$ and noticeably $Twist^{+}$ at 54 PDs. The rNA population was $Slug^{+}$, $Snail^{+}$ and $Twist^{+}$, but $P75^{-}$ when cultured to 12 PDs. With the fibronectin selected clonal populations the rFNA clone expressed all the neural crest markers and maintained them all until 119 PDs (figure 2.17b). The rNA clone also

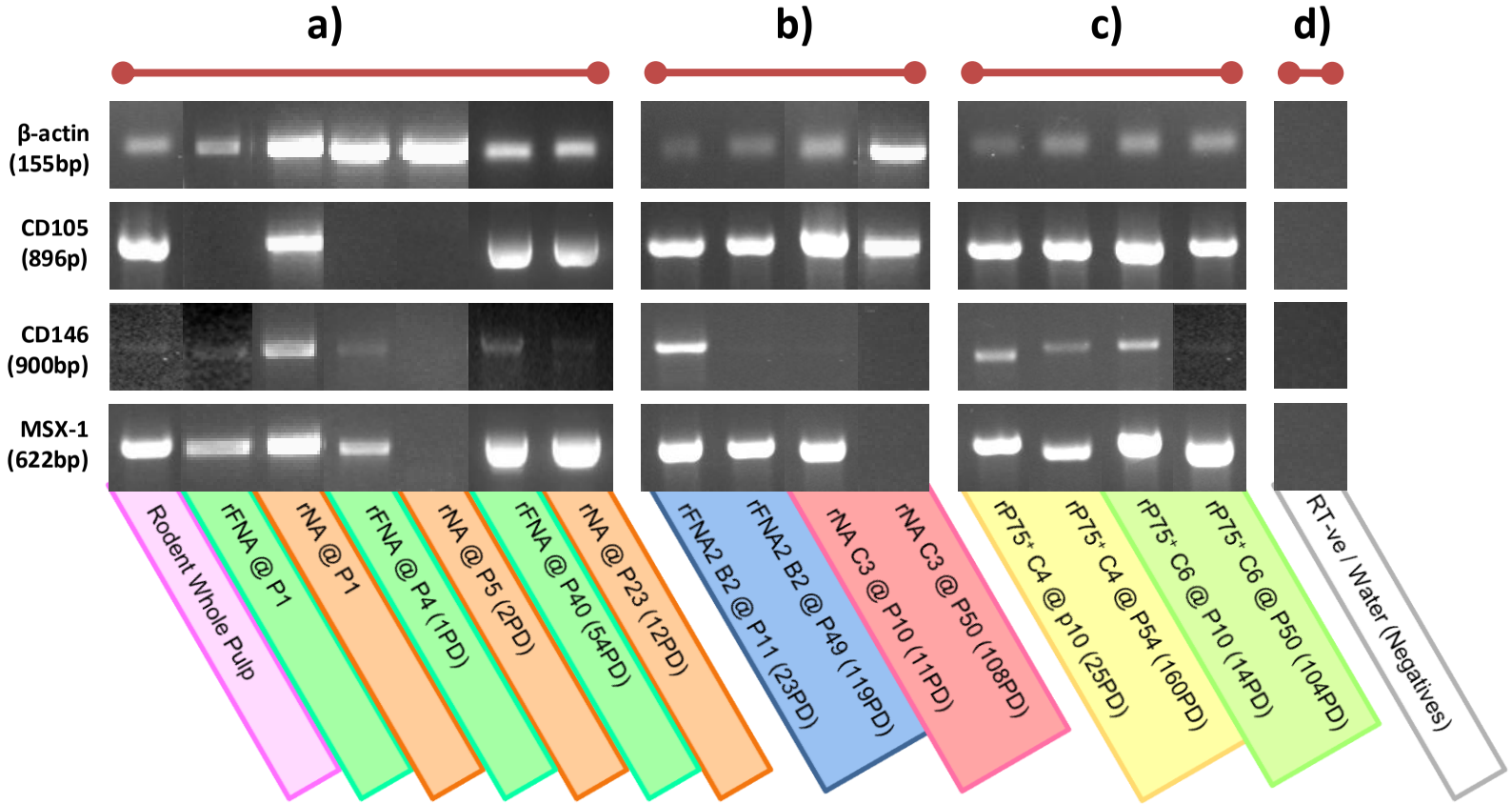



**Figure 2.16:** Mesenchymal markers expressed in rodent whole dental pulp and early fibronectin selected heterogeneous populations (a), fibronectin selected early clonal cultures (b) and rP75$^{+}$ sorted clones (c). Negative controls had no markers expressed (d).

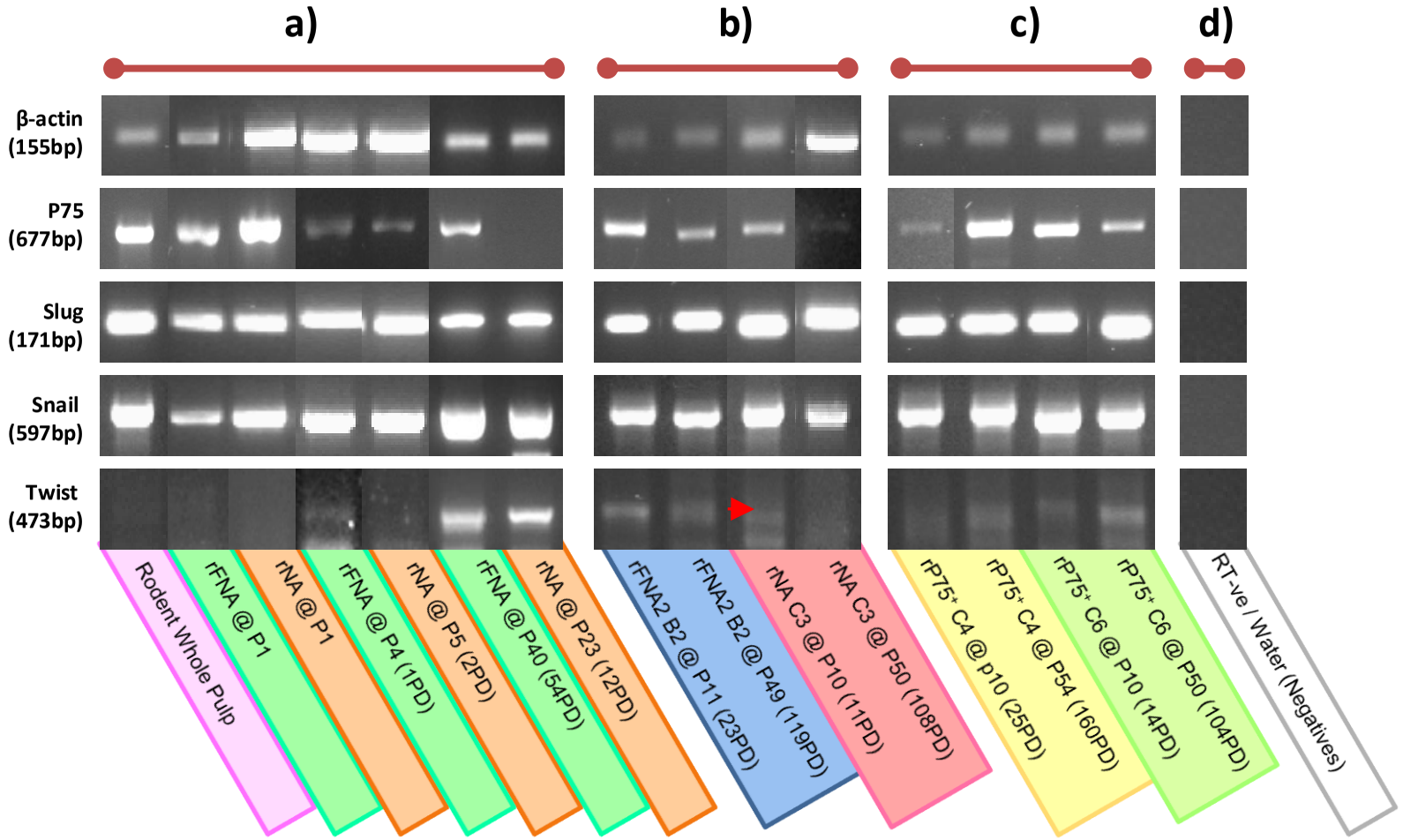



**Figure 2.17:** Neural crest markers expressed in rodent dental pulp heterogeneous populations, Twist appeared at later culture (a), fibronectin selected early clonal cultures (b), and all rP75$^{+}$ sorted clones (c) . Negative control samples had no markers expressed (d).

expressed all the neural crest markers at 11 PDs although it became Twist$^{-}$ with weak P75$^{+}$ expression at 108 PDs. The two rP75$^{+}$ selected clones C4 and C6 exhibited all the neural crest markers and maintained them into later culture at 160 and 104 PDs respectively (figure 2.17c). The water and RT-negative experimental controls had no expression of neural crest markers (figure 2.17d).

### 2.3.7.3. Embryonic Markers of Rodent DPSCs

The rodent dental pulp cells were consistently Oct4a$^{-}$ and Nanog$^{-}$ even after selection of progenitor cell populations by fibronectin or P75 sorting (figure 2.18). With regards to the other embryonic makers, the heterogeneous cell populations were TERT$^{+}$ and TR$^{+}$ even after fibronectin selection and maintained them into later culture (rFNA at 54 PDs and rNA at 12 PDs) (figure 2.18a). Selection of homogenous populations, the rFNA and rNA clonal populations were TERT$^{+}$ and TR$^{+}$ and were maintained into later culture (rFNA at 119 PDs and rNA at 108 PDs) (figure 2.18b). By sorting into rP75$^{+}$ clonal populations, clones C4 and C6 were TERT$^{+}$ and TR$^{+}$, with clone C4 changing to TERT$^{-}$ and TR$^{+}$ at 160 PDs and clone C6 remaining TERT$^{+}$ and TR$^{+}$ at 104 PDs (figure 2.18c). The water and RT-negative experimental controls had no expression of embryonic markers (figure 2.18d).

### 2.3.7.4. Early Developmental Markers of Rodent Progenitor Cells

Rodent dental pulp cells appear consistently Jagged1$^{+}$ and Jagged2$^{-}$ even with selection of progenitor cell populations by fibronectin or P75 sorting (figure 2.19). With regards to the remaining developmental markers the heterogeneous pulp populations were Notch1$^{+}$, 2$^{+}$ and 3$^{+}$ (figure 2.19a). Following fibronectin selection and after colonies were allowed to merge, the Notch1 expression for the rFNA

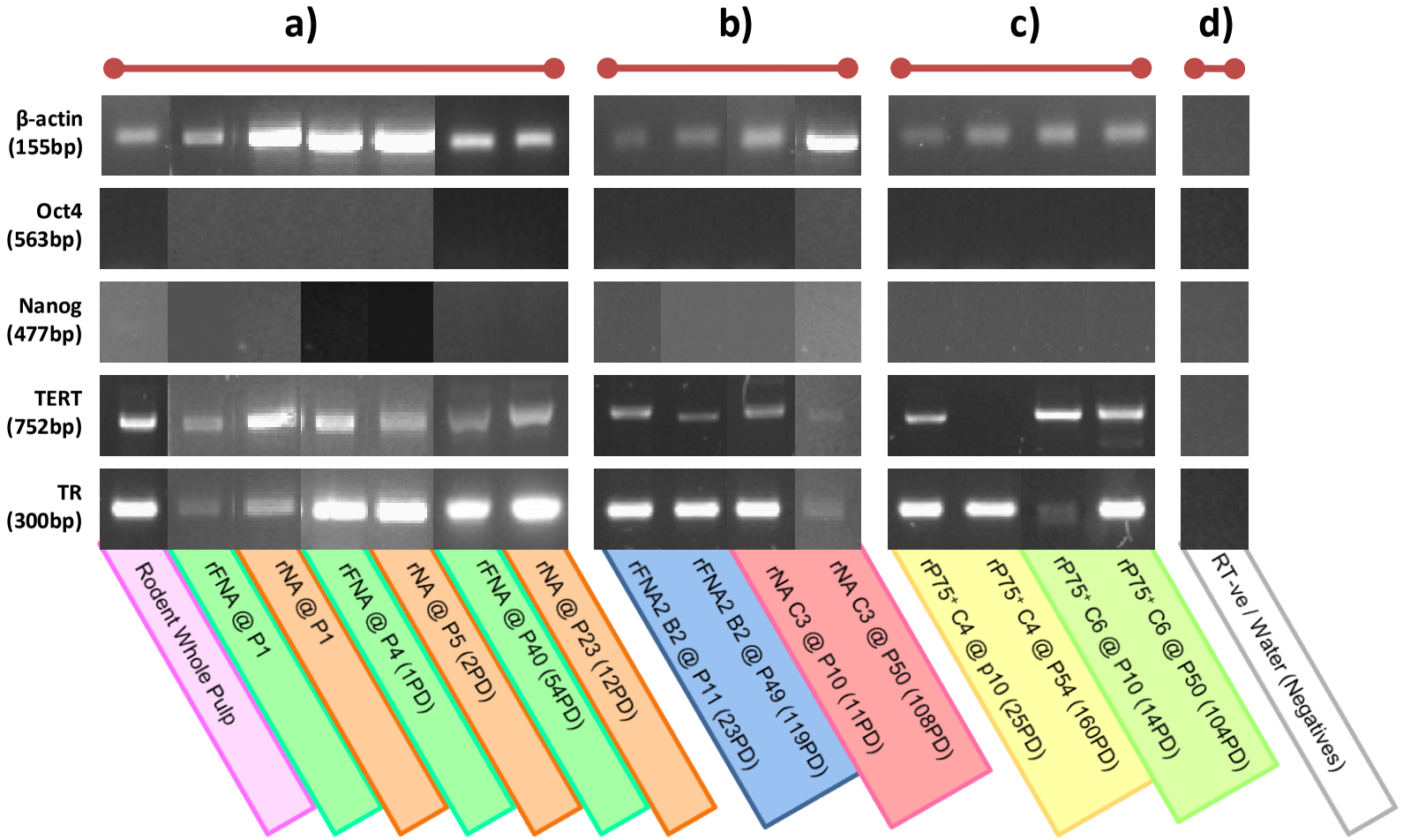



**Figure 2.18:** Embryonic markers TERT and TR expressed in rodent dental pulp heterogeneous populations (a), fibronectin selected clones (b), and rP75$^{+}$ sorted clones (c). Negative controls had no markers expressed(d).

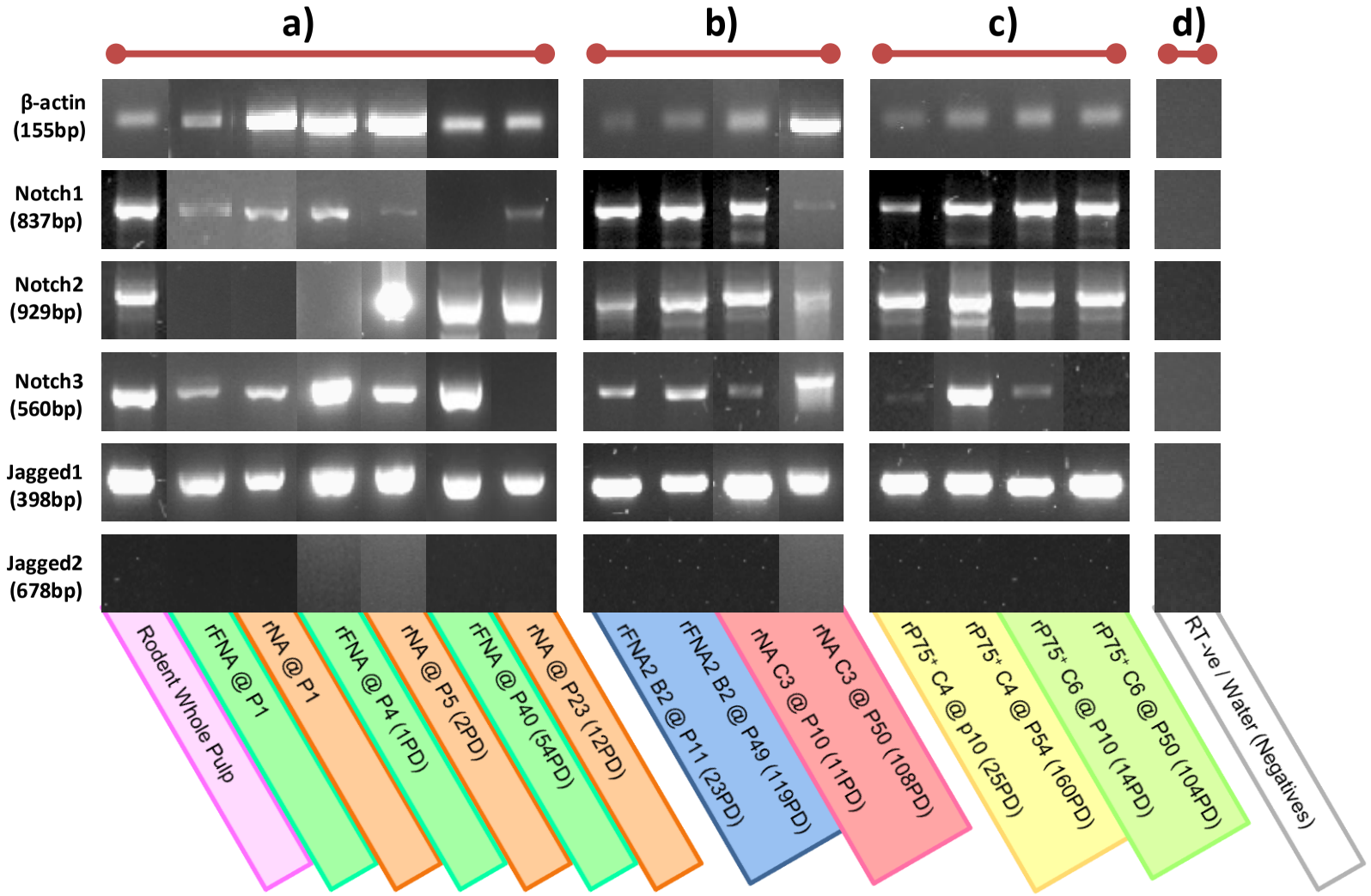



**Figure 2.19:** Developmental markers Notch1-3 and Jagged1 expressed in rodent pulp heterogeneous populations (a), fibronectin selected clones (b) and rP75$^{+}$ sorted clones (c). Jagged2 was not expressed in all samples and negative controls (d) had no marker expressed.

became weaker with longer culture and by 54 PDs became Notch1$^{-}$. Whereas the rNA population maintained weak Notch1$^{+}$ at 12 PDs. Notch2 was not expressed initially after fibronectin selection but expressed at later culture: rFNA at 54 PDs and rNA after just 2 PDs, both were Notch2$^{+}$. Notch3 expression was maintained in the heterogeneous rFNA and rNA populations, rFNA maintaining Notch3$^{+}$ to 54 PDs and rNA became Notch3$^{-}$ by 12 PDs.

When using fibronectin selected clonal populations the homogenous cell populations exhibited a more constant expression of all the developmental markers. The rFNA at 119 PDs and the rNA at 108 PDs both of which maintained expression of Notch1$^{+}$, 2$^{+}$ and 3$^{+}$ (figure 2.19b). Similarly the rP75$^{+}$ sorted clones C4 and C6 were also Notch1$^{+}$, 2$^{+}$ and 3$^{+}$ (figure 2.19c). These were maintained at 160 PDs for clone C4 and 104 PDs for clone C6. The water and RT-negative experimental controls had no expression of developmental markers (figure 2.19d).

## 2.4. Discussion

The dental pulp contains multiple progenitor cell populations of differing growth kinetics and stem cell markers when cultured *in vitro*. Isolated progenitors were proliferative, exhibited high growth kinetics and expressed multipotent and pluripotent gene markers. They also formed clonogenic colonies akin to somatic cells like the colony forming units-fibroblasts (CFU-F) of BMSC (Huang et al. 2009).

Long-term culturing of human pulp progenitors, particularly the hFNA clonal populations, led to the loss of some multipotent and embryonic markers possibly due to differentiation of the progenitors with culturing. The progenitors yielded from the rodent dental pulp maintained both multipotent and some embryonic markers (absence of Nanog and Oct4). This was possibly due to it being derived from continuously erupting rodent incisors with unclosed tooth apicies, where the dental pulp are thought to contain more immature progenitor populations. This discrepancy between the human and rodent progenitors highlights the species differences with more immature progenitors found in the rodent teeth and more mature cells in the human teeth.

The human dental pulp has previously been shown to contain a small fraction of neural crest cells, possibly derived from tooth development when cells migrate from the cranial neural crest, but also suggested to invade the pulp from residual neural crest cells that have remained inside adult tissues (Chai et al. 2000; Cho et al. 2003; Smith et al. 2005). Initial isolated $hP75^{+}$ cells were non-proliferative even after four repeats of the process. The whole pulp cell population was then cultured for one passage to expand cell

numbers to ensure there were enough $P75^{+}$ cells for MACS isolation which then produced a proliferative population.

Different isolated progenitor cell populations from the dental pulp were morphologically dissimilar to each other. Fluorescence staining of rFNA cells revealed a fibroblast-like population with multiple cellular projections, different to the oblong-like rNA population of cells. The $rP75^{+}$ MACS population of cells were stellate and half the size of the rFNA, rNA and $rP75^{-}$ populations with a smaller area of cytoplasm. As neural crest cells are an earlier cell type, which are more embryonic-like and less differentiated, it was suggested that these $rP75^{+}$ cells were more immature (Chai et al. 2000; Cho et al. 2003; Deng et al. 2004). The $rP75^{-}$ cells were larger in size compared with the $rP75^{+}$ cells and had varying morphologies, suggesting all the remaining cell types of the dental pulp were separated from the more homogenous population of the $rP75^{+}$ cells. These results suggest the use of different isolation methods for selecting progenitors from the dental pulp had isolated distinct progenitor populations. Sorting the progenitor populations with fibronectin or MACS had made a more morphologically homogenous population of cells, which may be of more value for tissue engineering. Future work may consider pre-treating the glass cover-slips with fibronectin to assist cell adhesion which may have an effect on cell morphology (Jones and Watt 1993).

Different populations of dental pulp progenitors yielded diverse proliferation rates which may be not ideal for translational medical treatments. However all the populations were capable of sustained growth kinetics, this highlights the presence of distinct progenitor populations which may be selected clonally for a more homogenous population for tissue engineering/clinical therapy development. The CFE of the rFNA

were significantly higher than the rNA populations and the rP75$^{+}$ was shown to be able to produce three times the CFE compared with rP75$^{-}$. The hFNA was less proficient in forming colonies although the CFE was higher in comparison to the hNA it was not significantly greater. The fibronectin preferential selection isolated a more immature cell type based on the functionality of the $\beta_1$-integrins and that the neural crest P75$^{+}$ population possessed embryonic-like cells. As immature progenitors are more proliferative in comparison to mature cell types it would have greater capacity to form colonies (Huang et al. 2009).

After investigating the PDs with qualitative comparisons, it appears that the DPSCs had greater proliferation compared with the BMSCs. It has been previously reported that BMSCs are capable of only 30-50 PDs (Huang et al. 2009) whereas the progenitors derived from hFNA, hNA, rFNA, rNA and rP75$^{+}$ were able to produce higher PDs. The mixed population of hFNA appeared to have a lower PD compared with the mixed hNA with a plateau in PDs lasting for over 100 days before the PDs increased. As the population of cells were heterogeneous it may be the cause of the plateau in the PDs. The isolated progenitors in the *in vitro* environment would have been of different states of maturity, differentiation potential and perhaps quantity of SCAPs. Had the more mature and differentiated cell fraction gradually become senescent and apoptotic, the more immature progenitors would have proliferated and increased the PDs. Conversely the mixed rFNA population had higher PDs compared with the mixed rNA populations.

The rodent progenitors were more competent in forming colonies and doubling their populations due to species differences, the continuously erupting rodent incisors suggested a higher amount of progenitors compared with the more static and mature

human tooth (Harada et al. 1999; Ohshima et al. 2005). The extracted adult human teeth would have been fully mature with a closed apex population of apical cells from the time the crown was fully developed (Suzuki et al. 2002; Zeichner-David et al. 2003). With an immature human tooth it has been well established that there are stem cells of the apical papilla (SCAP) shown to be 2-3 times more proliferative than DPSCs (Sonoyama et al. 2008). Dependant on tooth maturity during extraction this may have an imperative effect on the growth kinetics of the human isolated progenitors (Lei et al. 2011). With the rodent incisors the corresponding SCAP population, the apical bud cells (ABCs), composes of stellate reticulum surrounded by basal epithelial cells allowing for continuous ameloblast production and enamel formation (Fang et al. 2009). These ABCs may have contributed to the higher CFE and proliferative rates, as seen with the rodent isolated progenitors.

By selecting the more proliferative clones that had formed colonies early in the culture the selected cells and its negatively selected population of progenitors yielded a more sustained increase in PDs compared with the heterogeneous populations. Although some clones still experienced a lag in doubling, it may again be due to varied differentiated states of the progenitor cells within the population where the more mature subset of cells senesced before the immature cells proliferated to increase the PDs (or maybe differentiate). It was possible that different stimuli were being experienced by the individual cells of a colony where the central cells may not receive same signals the outer/distant cells experience. This may cause differing proliferation rates and maybe directed into different development routes when undergoing spontaneous differentiation. This growth-lag-growth PD pattern had also been seen in previous studies (Waddington et al. 2009). Another point to note is that the human patients of varying ages, sex and

condition of health may contribute to decreased growth kinetics (Ma et al. 2009; Wang et al. 2010) which may also explain the differing PDs with hFNA clones taken from two different patients (figure 2.6).

Distinct populations of progenitors resided in the dental pulp with the most immature populations being the hFNA clones in human teeth and the rFNA and rP75$^{+}$ clones in rodent incisors, these populations maintained multipotent and some embryonic-like markers particularly during early *in vitro* culture. It was noted that clonal populations in both human and rat progenitor populations maintained multipotent and typical embryonic markers. This correlates with the growth kinetics data where the selection of a proliferative colony may have chosen a more immature population of progenitors (e.g. SCAPs/ABCs) when compared to a heterogeneous cell population which may contain more mature cells (Lei et al. 2011). The PCR results were conducted in separate experiments due to the sheer number of markers that were investigated and that the samples were collected at separate "early" and "late" culture time points. Ideally the samples should have all the markers investigated in one experiment and with comparison with the early and late cultures, but this was not possible during this PhD study.

The human and rodent dental pulp was found to harbour cells expressing mesenchymal markers. The hFNA, hNA, rFNA and rNA isolates maintained expression of mesenchymal markers during early culture (20-50 PDs) but at later culture lost the majority of markers in the hFNA, hNA and rNA populations except for the rFNA, which maintained marker expression. The hFNA and hNA progenitor cells may have become more mature and more differentiated with culture losing its multipotency markers.

The rNA were negatively-selected cells from the fibronectin selection process, a more mature population compared to the cells with more functional $\beta_1$-integrins that were the rFNA. The rFNA possibly harboured more immature progenitors on the basis of the continuously erupting incisors (the immature DPSCs and ABCs mentioned earlier). The $P75^+$ populations also maintained mesenchymal marker expression even into later culture. This constant expression may be due to the fact that the ectomesenchymal tissues were derived from the neural crest (Chai et al. 2000) and it may also differentiate into mesenchymal lineages in culture for mesenchymal marker expression.

Ectomesenchymal cells were suggested to be of neural crest origins derived from neural folds (Blentic et al. 2008; Weston et al. 2004) which explains the expression of neural crest markers found in the early cultures of FNA, NA, $P75^+$ and $P75^-$ progenitor populations. In later culture of the hFNA, hNA and $hP75^-$ populations these neural crest markers were absent as the cell populations may have differentiated into more mature cell lineages with the lack of immature progenitors (SCAPs). However, rodent isolated populations (rFNA, rNA and $rP75^+$) were able to maintain neural crest markers possibly due to the immaturity of the available dental pulp progenitors (ABCs).

The dental pulp contained embryonic-like and undifferentiated progenitor cell populations. The human dental pulp expressed pluripotent cell markers Oct4a and Nanog but lacked the vital self-renewal expression of telomerase (hTERT) despite the RNA binding component (hTR) remaining present. The embryonic markers were present in hFNA/rFNA, hNA/rNA, and $rP75^+$ sorted populations (particularly during early culture) but not in the $hP75^+$/$hP75^-$ populations.

Although the hP75$^{+}$ population was a more immature cell type compared with the hFNA population, the absence of embryonic markers provided evidence of the selection of different progenitor populations that were less able to maintain an undifferentiated state. The expression of Oct4a is only present during early embryonic development where it is suggested to play a role in maintaining pluripotency in ESCs (Lee et al. 2006; Stefanovic et al. 2009) while Nanog expression prevents differentiation (Chambers et al. 2003), suggesting the hP75$^{+}$/hP75$^{-}$cells may have differentiated and lost its embryonic markers.

The expression of active telomerase (hTERT) is recognised as a major contributing factor for the continuous self-renewal or immortalisation of pluripotent stem cells (Fujii et al. 2008; Takahashi et al. 2007). Although the human progenitor cells lacked the self-renewal capacity, the presence of embryonic undifferentiated cells remained a valuable source of progenitors. Conversely, the rodent dental pulp lacked Nanog and Oct4 expression as similar to previous findings (Kerkis et al. 2006), suggesting the cells were more mature/differentiated and not as embryonic-like although the telomerase and its receptor (TERT and TR) were expressed for cell self-renewal (if active). This coincided with the continuous eruption of rodent incisors, the generation of new cells for the maintenance of the rodent dental pulp environment as it matures and forms new teeth tissues.

The dental pulp contained immature cell populations demonstrated by the expression of early developmental markers in progenitor isolates at early culture. The Notch signalling pathway plays a complex role in the neural tissue development, non-neural tissue

development, mediating tumourogenesis and was shown to be present in injured rodent teeth for new tissue formation (Mitsiadis et al. 2003; Weinmaster et al. 1991).

In the FNA/NA and P75 sorted populations, the expression of the developmental markers was present during early culture when the progenitors were more immature. With longer culture some of the Notch receptors were lost in the hFNA and hNA populations (Notch2 and Notch1 respectively). It has been suggested that Notch1 plays a role in inhibiting odontoblast differentiation (Zhang et al. 2008) and Notch2 is commonly expressed in undifferentiated cells, during tissue turnover, during the formation of odontoblasts and at sites of mineralisation. It is down-regulated in the pulp by the influx of TGF-β1 during dentine repair (Mitsiadis et al. 1999; Mitsiadis et al. 2003).

The absence of Notch, mesenchymal and embryonic markers in the hFNA and hNA cultures suggested the progenitors became more mature and differentiated with longer culture. For the rodent progenitors (rFNA/rNA/rP75$^{+}$) Notch1 and 2 were expressed during later culture which possibly indicated a higher proportion of immature progenitors (e.g. ABCs), enabling the continued ability to express early developmental markers with longer culture. The group had previously shown that these progenitors, unlike the human progenitors, were more immature by maintaining the expression of immature cell markers such as the neural crest markers into later culture, suggesting these cells were more capable in maintaining an undifferentiated state.

Notch3 was consistently expressed in both human and rodent dental pulps. Notch3 expression was found in perivascular cell groups which correlated with expression of vascular marker CD146, suggesting the progenitor cells arose from the developing blood

vessels and the Notch3 expression may also be indicator of differentiation normally seen during dentine repair (Gronthos et al. 2000; Lovschall et al. 2005).

The complex relationship between the Notch receptors expression is a delicate process for maintaining the progenitor cell differentiation state and tissue development. The expression of both differentiation and undifferentiated markers suggests of varying potential of progenitors found in the dental pulp with some cells more immature than others. As the progenitors were removed from its dental niche, there is loss of micro-environmental cues of the extracellular matrix known to have drastic implications on regulating the differentiation of resident progenitor cells. This may explain the variable markers of differentiation expressed during *in vitro* culturing (Fuchs et al. 2004; Nishikawa and Osawa 2007; Scadden 2006; Smith et al. 1994; Spradling et al. 2001).

The ligands Jagged1 and 2 were found in human pulp and only Jagged1 was found in rodent pulp. These ligands had been suggested to have a role in inducing the Notch pathway to increase proliferation and migration as commonly seen in tumourogenic tissues (Phillips et al. 2007; Wang et al. 2009; Yustein et al. 2010) therefore its expression may explain the proliferative capacity of the progenitor cells. This was especially true for the proliferative rodent ABCs within the dental pulp although this has yet to be clarified.

In summary this chapter isolated distinct populations of progenitors from within the dental pulp that expressed immature stem cell markers. Two different isolation methods were utilised, first being the selection of immature cells containing more functional $\beta_1$-integrins that bind to fibronectin and the second was the selection of more embryonic-

like neural crest $P75^{+}$ cells. Each selected (and negatively selected) population demonstrated sustained increase of growth kinetics with the expression of multipotent and the suggested pluripotency markers which suggested the selection of immature progenitors in each population.

The loss of multipotent or pluripotent markers with long term *in vitro* culture was observed which further supports previous literature findings (Patel et al. 2009; Yu et al. 2010). The growth kinetics and stem cell markers displayed by the side population of cells had also been demonstrated in previous studies (Iohara et al. 2009) which are further evidence for the existence of multiple progenitors residing in the dental pulp.

For the human progenitors the lack of the more immature and proliferative cells (e.g. the SCAP), the clonal culturing selected a more homogenous population of immature cells from the more mature cells. Overall the results imply the use of early isolated clonal cultures for future experiments would be more ideal such as determining the progenitor cells' differentiation potential and behaviour to external stimuli as during a dentine injury response.

# Chapter 3:

# Differential Potential of Isolated Dental Pulp Stem Cells

## 3.1. Introduction

Stem cell differentiation is vital for the maintaining/repair of the surrounding niche environment. Determining the differentiation behaviour provides insight to stem/progenitor cell potential. Immature cells may express multipotent or pluripotent genotypic markers but their potential is determined by its ability to differentiate into another phenotype when induced by their local environment, making them valuable for tissue engineering studies.

Of the dental progenitor populations much work on multipotency has been carried out on stem cells of the pulp (DPSCs), human exfoliated deciduous teeth (SHED) cells, periodontal ligament (PDLSC) and immature papillae (SCAP) populations, particularly for the differentiation into multipotent mesenchymal lineages (Fang et al. 2009; Fujii et al. 2008; Seo et al. 2004; Shi et al. 2005; Sonoyama et al. 2008). The identification of progenitors in the human post-natal dental pulp (the DPSCs) has been first proven by Gronthos and co-workers (2005), where the whole dental pulp contained progenitors that were similar to bone marrow stromal cells (BMSCs), expressing putative MSC markers such as Stro-1 and perivascular marker CD146 but not the haematopoietic markers such as CD34 (Shi et al. 2005). However, dental tissue is unlike bone as it does not undergo continuous remodelling and it develops from the ectomesenchyme which is suggested to derive from neural crest cells (Huang et al. 2009).

Although similar in cellular morphology, it has been suggested the DPSCs and bone marrow stem cells (BMSCs) represent different progenitor cell populations. DPSCs illustrate a higher colony forming efficiency (CFE) and a different differentiation potential when compared with BMSCs. DPSC clones cultured in mineralising media demonstrated to form calcium nodules but BMSCs formed mineralised sheets and lipid droplets (Gronthos et al. 2000; Shi et al. 2005).

Mesenchymal stem cells (MSCs) have demonstrated its differentiation into osteogenic, chondrogenic, adipogenic, myelosupportive stroma, myogenic and neurogenic lineages as indication of its multipotent differentiation (Caplan 1991; Ferrari et al. 1998; Huang et al. 2009; Kadar et al. 2009; Pittenger et al. 1999; Tuli et al. 2003). Studies using BMSCs showed differentiation into osteoblasts, adipocytes, chondrocytes, myocytes and even neural cells (Pittenger et al. 1999; Rastegar et al. 2010; Stocum 2001). With dental stem cells, the human PDLSC, SHED and DPSCs were shown to be able to differentiate into mineralising cell types such as odontoblasts, osteoblasts, adipocytes, myocytes and neuronal cells (Fujii et al. 2008; Ikeda et al. 2010; Kadar et al. 2009; Laino et al. 2006; Wei et al. 2008), although none of the human experiments used the same isolated population for differentiation into multiple lineages compared with studies when using rodent dental stem cells (Waddington et al. 2009; Yang et al. 2007a; Yang et al. 2007b).

This chapter aims to further expand the knowledge of the potential of a specific human clonal population of DPSCs. This will be determined by its differentiation

into the multiple mesenchymal lineages: osteocytes, chondrocytes and adipocytes. Previous literature on human DPSC has never attempted differentiation on all three lineages with the same clonogenic cell population. Studies that utilised a non-clonal population yielded results where the progenitors strongly formed mineralised tissues but were weaker in their ability to differentiate into adipogenic or chondrogenic tissues (Djouad et al. 2010) which may be due to the presence of the more differentiated/established populations of progenitors present.

By utilising the hFNA clonal population of cells demonstrated previously to be proliferative and expressed multipotent gene markers (figure 2.7 and table 2.3), these will be cultured in differentiation media before determining their phenotypic and genotypic changes. The results attained would determine if a homogenous population of immature DPSCs has the potential to differentiate into multiple mesenchymal lineages which would illustrate the potentiality of the dental pulp progenitors, a valuable asset for study of dental progenitors in dentine repair therapies and for novel regenerative therapy development with tissue engineering.

## 3.2. Materials and Methods

### 3.2.1. Cell Source

The isolated DPSC clonal population, hFNA3 clone C4 expresses multipotent markers at 24 PDs (figure 2.12 – 2.15). This clone was cultured in adipogenic, chondrogenic and osteogenic induction media to ascertain the potential of the isolated DPSCs to differentiate into mesenchymal lineages.

### 3.2.2. Culture Media

Culture medium was composed of alpha-modification Minimum Essential Medium (αMEM) pre-supplemented with ribonucleosides and deoxyribonucleosides (Gibco, Invitrogen, UK). This was supplemented with hi-FCS, L-glutamine, L-ascorbate 2-phosphate and antibiotics/antimycotics as previously described in section 2.2.2.

#### 3.2.2.1. Adipogenic Differential Media

Adipogenic supplements were prepared as follows: the dexamethasone was dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, UK) for a concentration of 100 µM before a 1:100 dilution in the αMEM (Gibco, Invitrogen, UK) for a final working concentration of 1 µM; indomethacin (Sigma Aldrich, UK) was dissolved in DMSO for a concentration of 100 mM, diluted 1:1000 in the αMEM for a working concentration of 100 µM. The 3-isobutyl-1-methyl-xanthine (IBMX) stock was dissolved in 0.35 M potassium hydroxide (KOH) (both Sigma Aldrich, UK) to produce a 100 mM concentration and further diluted in αMEM at 1:1000 for a working concentration of 100 µM. Insulin (Sigma Aldrich, UK) was diluted in αMEM at 10 mg/mL stock before further diluting in the culture medium at 1:1000 for a 10 µg/mL final concentration. The stocks were frozen at -20℃ immediately

after preparation and thawed to room temperature prior addition into the culturing medium.

Adipogenic media was based on supplementations previously used in the literature (Davies et al. 2010). The adipogenic induction medium (AIM) was composed of αMEM supplemented with 10% hi-FCS (Gibco, Invitrogen, UK), 10 μg/mL insulin, 1 μM dexamethasone, 100 μM indomethacin and 100 μM IBMX with antibiotics/antimycotics (as section 2.2.2). Adipogenic maintenance medium (AMM) was prepared using αMEM supplemented with 10% hi-FCS, 10 μg/mL insulin and antibiotic/antimycotics (as section 2.2.2).

#### 3.2.2.2. Osteogenic Differential Media

The basal medium αMEM was supplemented with 20% hi-FCS, 10 nM dexamethasone, 100 μM L-ascorbate 2-phosphate, 100 μM β-glycerolphosphate (additives from Sigma Aldrich, UK) and antibiotics/antimycotics (as section 2.2.2).

The dexamethasone and β-glycerolphosphate were prepared as follows: the dexamethasone was dissolved in DMSO for a concentration of 1 μM before dilution 1:100 in the αMEM for a final working concentration of 10 nM and β-glycerolphosphate was dissolved in phosphate buffered saline (PBS) at a 1 M stock which was diluted 1:100 in the αMEM for a final working concentration of 10 mM.

#### 3.2.2.3. Chondrogenic Differential Media

The medium used was the NH stem cell media ChondroDiff medium (Miltenyi Biotec, UK). The medium was thawed and supplemented with

antibiotics/antimycotics (as section 2.2.2) and then divided into aliquots for storage at -20℃. The components of the chondrogenic differentiation medium is not disclosed by the supplier, but believed to be similar to chondrogenic medium mentioned in literature which contained insulin, transferrin, selenium, L-ascorbate 2-phosphate and TGF-β1 (Davies et al. 2010).

### 3.2.3. Differentiation Induction Procedures

Differentiation of cultures into adipogenic, osteogenic and chondrogenic lineages is listed below. A day 0 control was also performed consisting of DPSCs plated into 2× 6-well plates (Greiner Bio-One, UK). These were cultured in culture medium until confluent and the cells were immediately used for total RNA extraction and RT-PCR (section 3.2.6).

#### 3.2.3.1. Adipogenic Differentiation

The adipogenic differentiation was performed in 2× 6-well plates. One plate had 2 or 3 sterile 13 mm diameter sterile glass cover-slips (Raymond A Lamb, Thermo Scientific, UK) placed at the base of each well which allowed cells to adhere on the surfaces for histology staining. The second plate had no cover-slips and was for total RNA extraction for RT-PCR (section 3.2.6). A repeat of the above set-up was cultured in normal culture medium as the control cultures.

A concentration of $5x10^4$ cells/well was seeded into 2 different 6-well plates with 2 mL of culture medium and cultured until 90-100% confluency. The medium was replaced with AIM and cultured in 37°C and 5% $CO_2$ for 72 hrs before replacing with AMM for 24 hrs. The cells were treated with the AIM and AMM alternately for

4 rotations (total 16 days) before culturing the cells in AMM for a final 7 days, changing medium every 72 hrs. On day 23 the cells were processed for analysis. A repeat of the above set-up was cultured in normal culture medium as control cultures. The culturing process is based from previous literature (Davies et al. 2010) summarised in figure 3.1.

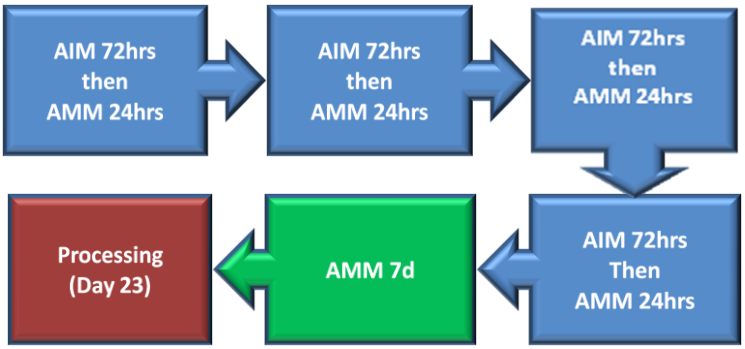



**Figure 3.1: Summary of the adipogenic differentiation culture procedure.**

### 3.2.3.2. Osteogenic Differentiation

Osteogenic differentiation was performed on 3× 6-well plates. Two plates had 2 or 3 sterile 13 mm diameter glass cover-slips placed at the base of each well where cells to adhered on the surfaces. One of the plates was used for histology staining and one plate was for immunocytochemistry (ICC). The third plate was for total RNA extraction for RT-PCR (section 3.2.6). A repeat of the above set-up was cultured in normal culture medium as control cultures.

A concentration of $5x10^4$ cells/well were seeded into 3 separate 6-well plates with 2 mL of culture medium and cultured until 90-100% confluency. The medium was replaced with the osteogenic mineralising medium and cultured in 37°C and 5% $CO_2$

with media changes every 48 hrs. On day 23 the cells were processed for analysis. A repeat of the above set-up was cultured in normal culture medium as control cultures.

#### 3.2.3.3. Chondrogenic Differentiation

The chondrogenic differentiation was cultured into 5 × 15 mL polypropylene conical base tubes (Falcon, UK) where 3 were used for IHC labelling and 2 were used for total RNA extraction for RT-PCR (section 3.2.6). Another 5 tubes of cells were cultured in normal culture medium as controls where 3 tubes were used for IHC and 2 were used for total RNA extraction.

In 5 different 15 mL polypropylene tubes, a single cell suspension with $2.5x10^5$ cells in 1 mL was centrifuged at 150 ×g for 5 mins to produce a cell pellet. The medium was aspirated and the cell pellet resuspended with 1 mL ChondroDiff medium before centrifugation again and the supernatant carefully aspirated. To the pellet of cells, 1 mL of the ChondroDiff medium was carefully added to avoid disruption to the cell pellet.

The caps of the conical tubes were loosely screwed on and the pellets were cultured in 37°C with 5% $CO_2$ with fresh medium changes every 72 hrs up till 24 days of culture. A repeat of the above set-up was cultured in normal culture medium as control cultures. The pellet culture method was adopted as it has previously been shown to successfully demonstrate chondrogenic differentiation by various research groups (Davies et al. 2010; Dowthwaite et al. 2004).

### 3.2.4. Histology of Adipogenic and Osteogenic Cultures

For both adipogenic and osteogenic cultures and control cultures, one 6-well plate containing glass cover-slips was washed twice with PBS before fixed with 4% paraformaldehyde (PFA) for 10 mins at room temperature. The PFA was then removed and the fixed cells were washed twice with PBS ready for staining.

#### 3.2.4.1. Oil Red O Staining

The Oil red O (stock of 3.5 g/L dissolved in isopropanol) working stain was prepared fresh by mixing 4:6 ratio of water to oil red O stock. This was incubated at room temperature for 10 mins on a shaker before filtering through a Whatman™ grade 42 (2.5μm) filter paper. The adipogenic cultures and its controls were washed with 60% isopropanol for 5 mins with gentle agitation before aspirating and addition of 1-2 mL/well working oil red O solution for 10 mins at room temperature with gentle agitation. The stained cells were gently washed with water and then washed briefly with 60% isopropanol before washing with more water until the excess stain was removed. The samples were air dried before viewing using a light microscope.

#### 3.2.4.2. Alizarin Red S Staining

The osteogenic cultures and its controls in the 6-well plates were stained with 1-2 mL/well alizarin red S (20 g/L dissolved in distilled water, pH to 4.1-4.3 using 10% ammonium hydroxide) for 2-5 mins with gentle shaking. This was then gently washed with running water until the excess stain was removed, air dried and viewed under a light microscope.

### 3.2.5. Immunohistochemistry of Differentiated Cultures

#### 3.2.5.1. BSP Detection in Osteogenic Cultures

One osteogenic and control culture on 6-well plates containing glass cover-slips were washed twice with PBS and fixed with 4% PFA for 10 mins at room temperature. The PFA was then removed and the fixed cells washed twice with PBS and blocked in 1% BSA- tris-buffered saline (TBS) blocking buffer for 1 hr. Samples were then incubated with rabbit-raised anti-BSP (LF-87) IgG antibody (kindly donated by Dr Larry Fisher) diluted 1:100 in blocking buffer for 1 hr at room temperature. The cultures were then washed 5 times with TBS before treating with chicken-raised anti-rabbit-FITC (Santa Cruz) IgG antibody which was diluted 1:100 in blocking buffer for 45 mins at room temperature and kept in darkness. The excess antibodies were aspirated and the samples washed with TBS before the addition of the nuclear counter-stain bisBenzimide at 2.5 μg/mL in blocking buffer, incubated 30 mins at room temperature and kept in darkness.

Samples were then washed with TBS before the cells on the cover-slips were lifted off the 6-well plates with forceps. The cover-slips were then mounted using Fluorsave (Calbiochem, UK) onto a glass slide and left overnight in the dark at 4°C, before viewing the next day using a UV microscope (section 2.2.8) at 373 nm/456 nm (FITC) and 490 nm/520 nm (bisBenzimide). Wells treated with a non-immunogenic IgG isotype antibody (Sigma Aldrich, UK) instead of the primary antibody or with primary antibody exclusion (only blocking buffer) were used as experimental controls.

### 3.2.5.2. Chondrogenic Pellet Sectioning and 2B6 Antigen-Labelling

The 3 experimental and 3 control pellet cultures were washed once with PBS before fixed overnight in 3.7% neutral buffered formalin (in PBS) with gentle agitation. After careful aspiration of the formalin each cell pellet was carefully removed from the conical tube using a long thin spatula, then loosely wrapped in filter paper and inserted into embedding cassettes for automatic processing (Shandon Pathcentre). This process dehydrated the samples by passing it through increasing 70%, 90% and 100% concentrations of IMS. Samples were paraffin wax embedded and cut into 5 μm sections using a sliding microtome (Leica SM2400, Leica Microsystems, UK).

The sections were collected on poly-L-lysine coated glass slides (SuperFrost, Thermo Fisher Scientific, UK) and allowed to dry overnight at 60°C. The sections were deparaffinised and rehydrated by washing 10 mins with xylene, 5 mins with 70% IMS and 5 mins with water. Sections were circled with immiscible ink using a Super PAP Pen (Daido Sangyo, Japan) before treated with 0.15 U/mL chondroitinase ACII (SeikaGaku Kogyo Co., Japan) in Tris acetate, pH8, for 30 mins at 37°C to remove GAG chains and reveal the chondroitin-4-sulfate neo-epitopes. Samples were then washed with TBS before blocking with 1% BSA-TBS buffer for 30 mins.

A mouse raised anti-2-B-6 IgG monoclonal antibody (kindly donated by Professor B. Caterson, Cardiff University, UK) at 1:500 in blocking buffer was then added and incubated for 1 hr in room temperature. Samples were washed with TBS before the addition of goat raised anti-mouse IgG-FITC (Santa Cruz) at 1:200 dilutions in blocking buffer for 45 mins at 4°C and kept in darkness. Sections were washed with

TBS before addition of 2.5 μg/mL bisBenzimide in blocking buffer for 30 mins in darkness and at 4°C before washing again with TBS.

The sections were washed 5 mins with 70% IMS then 5 mins with xylene before the addition of Fluorsave (Calbiochem) for mounting with a glass coverslip. The samples were kept in darkness and at 4°C overnight before viewing using an UV microscope (section 2.2.8). The exclusion of the primary antibody or the use of a mouse $IgG_1$ isotype antibody (Sigma Aldrich, UK) as a substitute for the primary antibody was used as experimental controls.

### 3.2.6. RT-PCR of DPSC Differentiation

RT-PCR was performed on one plate of adipogenic and osteogenic cultures and 2 chondrogenic pellet cultures of differentiation (and its controls). As experimental controls, day 0 of culture DPSCs were used as a pre-differentiation negative control and cultures in normal culture medium were also used as negative controls alongside the differentiation cultures. Positive RT-PCR controls included Quantitative PCR Human Reference Total RNA (Agilent Technologies, UK), adipocyte cDNA provided by Dr. Marian Ludgate (Cardiff University, UK), osteocytes provided by Dr Bronwen Evans (Cardiff University, UK) and chondrocyte RNA provided by Dr Emma Blaine (Cardiff University, UK). RT-negatives and water negatives were used as the experimental controls and β-actin was used as the housekeeping gene for PCR.

Adipogenic, osteogenic and control culture total RNA were extracted using RNeasy spin column technology before quantification and RT-PCR (section 2.2.13.1 – 2.2.13.6). The chondrogenic differentiation (and control) pellets were lysed by

placing the pellets into a sterile eppendorf of 250 µL of Buffer RLT lysis buffer (Qiagen, UK) supplemented with 10 µL/mL of β-mercaptoethanol (Sigma-Aldrich, UK) and immediately homogenised. The lysate was used for RNA extraction, quantification and RT-PCR (sections 2.2.13.1 – 2.2.13.6). The PCR primers for adipogenic markers include lipoprotein lipase (LPL) and peroxisome proliferator-activated receptor-γ2 (PPARG2), osteogenic markers were osteocalcin (OCN) and bone sialoprotein (BSP), and chondrogenic markers used were collagen type II (Col2a) and Sox9. Primer sequences, melting temperatures and product sizes are summarised in table 3.1.

**Table 3.1: Human adipogenic, osteogenic and chondrogenic differentiation gene markers PCR amplification. β-actin was used as a housekeeping gene.**

| *Gene Product* | Human | | | |
|---|---|---|---|---|
| | ***Primer Sequence 5' - 3'*** | ***Annealing Temperature (°C)*** | ***Product Size (bp)*** | ***Source*** |
| Ostocalcin<br>*BGLAP* | Forward: 5'-GCAGGTGCGAAGCCCAGCGGTGCAGAG-3<br>Reverse: 5'-GGGCTGGGAGGTCAGGGCAAGGGCAAG- | 62 | 341 | Author's |
| Bone Sialoprotein 2 (BSP2)<br>*IBSP* | Forward: 5'-GGGCTATGGAGAGGACGCCACGCCTGG-3<br>Reverse: 5'-CGAGGTGCCCTTGCCCTGCCTTCCGGTC-3 | 62 | 340 | Author's |
| Sox9<br>*sox9* | Forward: 5'-GTGAACTGGCCACCCCGCGCCTTCCTA-3'<br>Reverse: 5'-CAGCCTTGCCCGGCTGCACGTCGGTTT-3' | 62 | 936 | Author's |
| Type 2 Collagen<br>*col2a1* | Forward: 5'-GGCTGGCAGCTGTGTGCAGGATGGGCA-3'<br>Reverse: 5'-GCGCCAGCAGGGCCAGTCCGTCCTCTT-3' | 62 | 930 | Author's |
| Lipoprotein Lipase (LPL)<br>*lpl* | Forward: 5'-GCTGGCATTGCAGGAAGTCTGACCAATAA<br>Reverse: 5'-GGCCACGGTGCCATACAGAGAAATCTCAA | 56 | 621 | Author's |
| Human Peroxisome Prolifer<br>*pparg2* | Forward: 5'-GCCATCAGGTTTGGGCGGATGCCACAG-3'<br>Reverse: 5'-CCTGCACAGCCTCCACGGAGCGAAACT-3' | 62 | 349 | Author's |
| β-actin<br>*actb* | Forward: 5'-AGGGCAGTGATCTCCTTCTGCATCCT -3'<br>Reverse: 5'-CCACACTGTGCCCATCTACGAGGGGT -3' | 65 | 480 | Dr. XQ Wei<br>(Cardiff University) |

## 3.3. Results

### 3.3.1. Adipogenic Differentiation of DPSCs

The hFNA3 C4 clonal population of DPSCs were cultured to confluency before induction. Consequently the initial cell morphology at confluence for both adipogenic cultures and control cultures were similar, but after 6 days of culture in adipogenic media the cells produced less matrix and became wider in morphology (figure 3.2a). In comparison, the control cells were long and narrow in morphology with cells as multi-layers and large areas of matrix secreted (figure 3.2b). At the end of the culture period at day 23, the cells in adiogenic media were highly confluent with multi-layer cells but appeared wider in morphology (figure 3.2c) when compared with the control culture. The control culture appeared the same as day 6, remaining highly confluent with overlaid cells and matrix secretions (figure 3.2d).

With oil red O staining after 23 days the adipogenic cultured cells demonstrated large stained clusters at areas of cell confluence and magnified images revealed the lipid droplets within the cells were stained (figure 3.3a). The control cultured cells when stained with the same oil red O revealed scarce lipid droplet production by the cells within the whole culture (figure 3.3b). Supporting the staining results, the RT-PCR results demonstrate the cells cultured in adipogenic culture expressed the adipogenic marker PPARG2 (although not LPL). The control cultures did not express any of the adipogenic markers and the positive control samples (Stratagene total RNA and adipocyte cells) were positive for the adipogenic markers (figure 3.4). In summary, the DPSCs cultured in adipogenic media were positive for the adipogenic stain and expressed one of the adipogenic gene markers, whereas the control cultures did not.

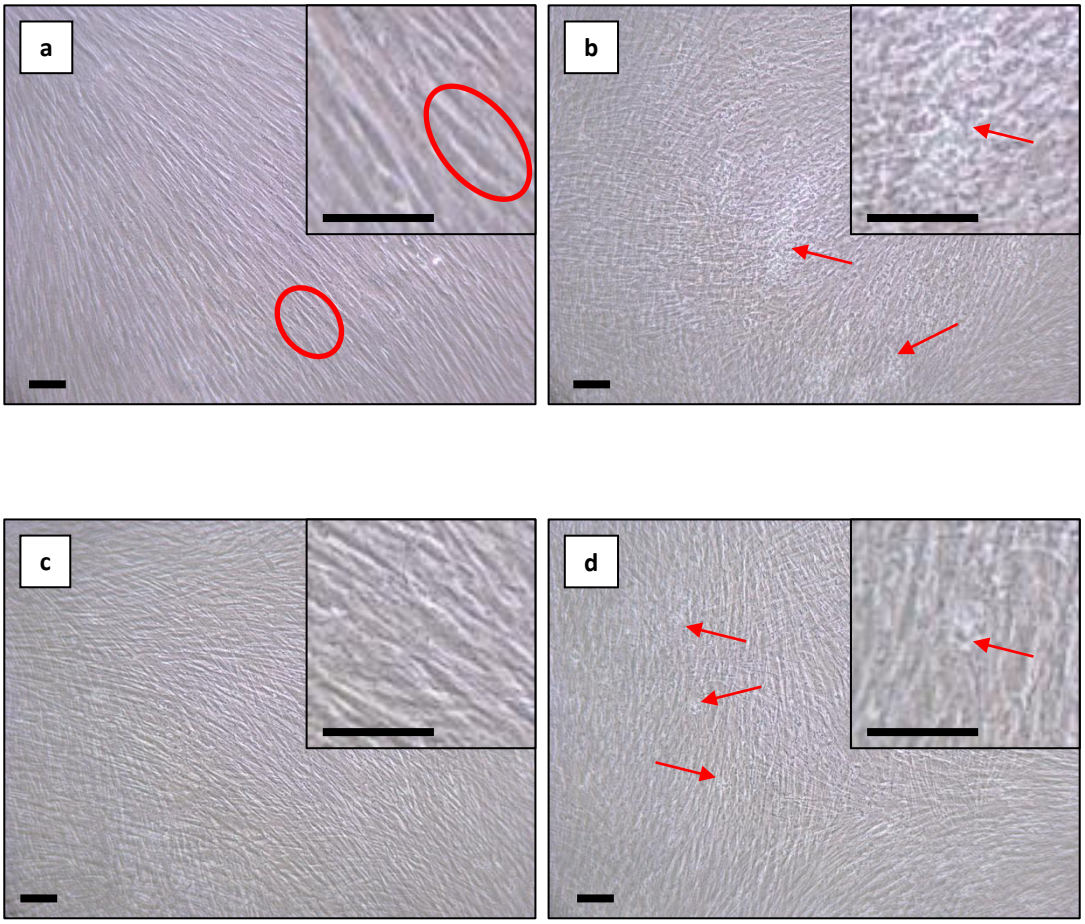



**Figure 3.2:** **Light microscopy of adipogenic induction and control cultures for 12 and 23 days. After 12 days of culture, the adipogenic cells appeared wider (circled example) and not secreted matrix (a), compared with the control cells which remained narrow and formed multi-layers with matrix secreted (b). On day 23, the adipogenic cells became highly confluent with overlaying cells, but maintained similar cell morphology (c). The corresponding control culture remained confluent, although the matrix secreted was more dispersed (d). ×10 magnification and insets are x30 magnification of the same image, arrows illustrates the matrix secretions and scale bar represents 100 µm.**

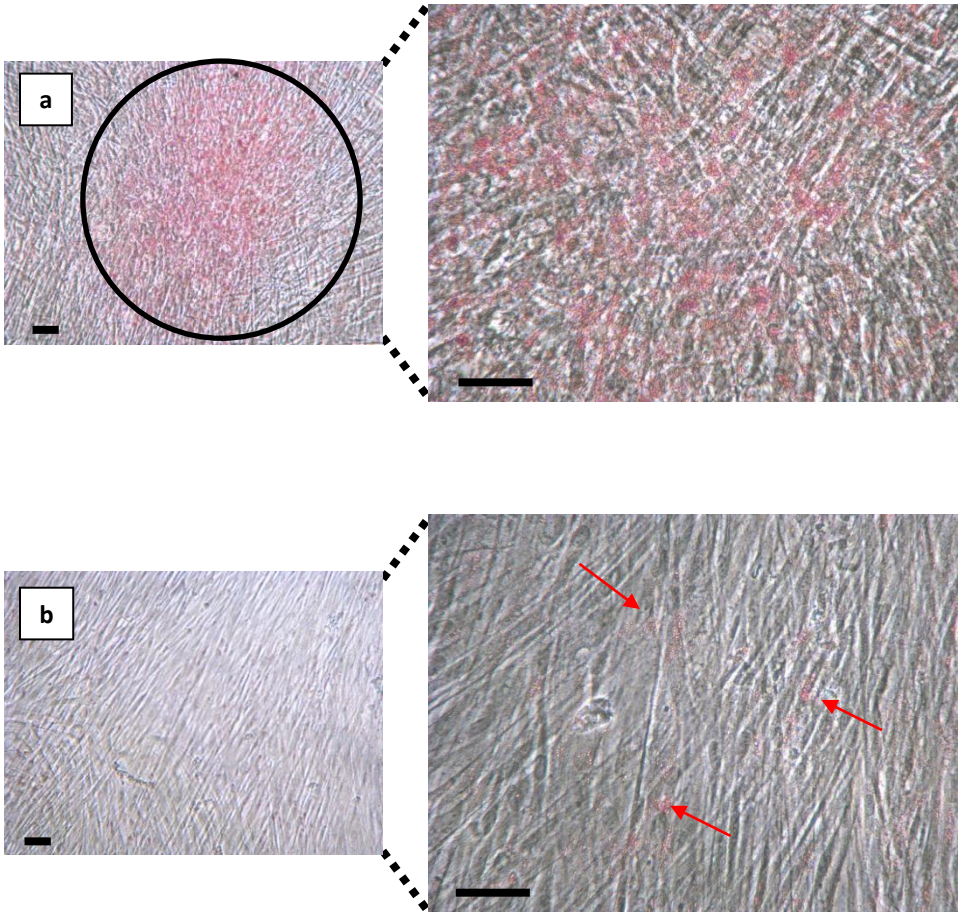
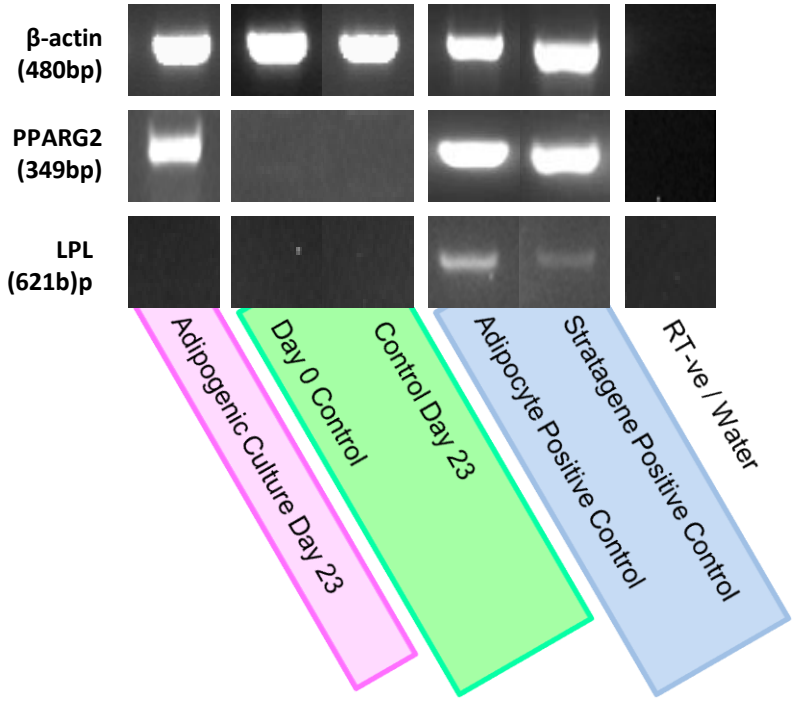



**Figure 3.3:** **Light microscopy images (×10) of Oil red O staining at 23 days of culture and a higher (×40) magnification image. The adipogenic induction culture (a) illustrates lipid droplets were formed by cells in the culture, and formation of clusters of lipid positive cells (circled) were found though out the adipogenic culture. The control culture (b) after staining with oil red O did not reveal significant levels of lipid droplets, although a few sparse droplets were found within some cells (some arrowed). Scale bar represents 50 µm.**




**<u>Figure 3.4:</u> Cells in adipogenic culture expressed PPARG2 only, whereas control cultures did not express any of the adipogenic markers. The markers were revealed to work in the positive control cDNA and all the samples were positive for β-actin housekeeping gene. The RT and water negatives remained blank.**

### 3.3.2. Chondrogenic Differentiation of DPSCs

Culture of DPSCs with the ChondroDiff chondrogenic medium caused the cell pellets to become rounded by day 3 (figure 3.5a) where the control cultures remained as pellets adhered to the bottom of the conical tube (figure 3.5b). By day 15 of culture the pellets in chondrogenic cultures appeared to become smaller and compact (figure 3.5c), while the control cultures lost its initial pellet appearance and became a mass of cells (figure 3.5d). By the end of the culture period, day 24 chondrogenic culture pellets appeared very small, rounded and compact (figure 3.5d) and the control cultures were irregular masses of cells (figure 3.5e).

These pellets when fixed, processed, sectioned and enzymatically digested were immunolabelled for 2B6 antigens which fragmented the pellet sections due to the repeated wash steps. The chondrogenic cultured pellets were shown to be rounded when it was sectioned and the pellets were positively labelled by the 2B6 antibody (figure 3.6a). The control cultured pellets were negative for 2B6 although there is an edging effect, a typical artefact of immunohistochemistry (figure 3.6b). The exclusion of the primary (2B6) antibody (figure 3.6c) and the use of an immunogenic isotype antibody (figure 3.6d) produced negatively labelled sections as experimental controls.

The pellets that were cultured in chondrogenic medium were shown to express chondrogenic markers Col2a and Sox9 with RT-PCR. The control cultures were negative for both of these markers whilst the Stratagene and chondrocyte cDNA positive controls were positive for both markers (figure 3.7). In summary the DPSC cells cultured in chondrogenic medium formed into tight pellets positive for the 2B6

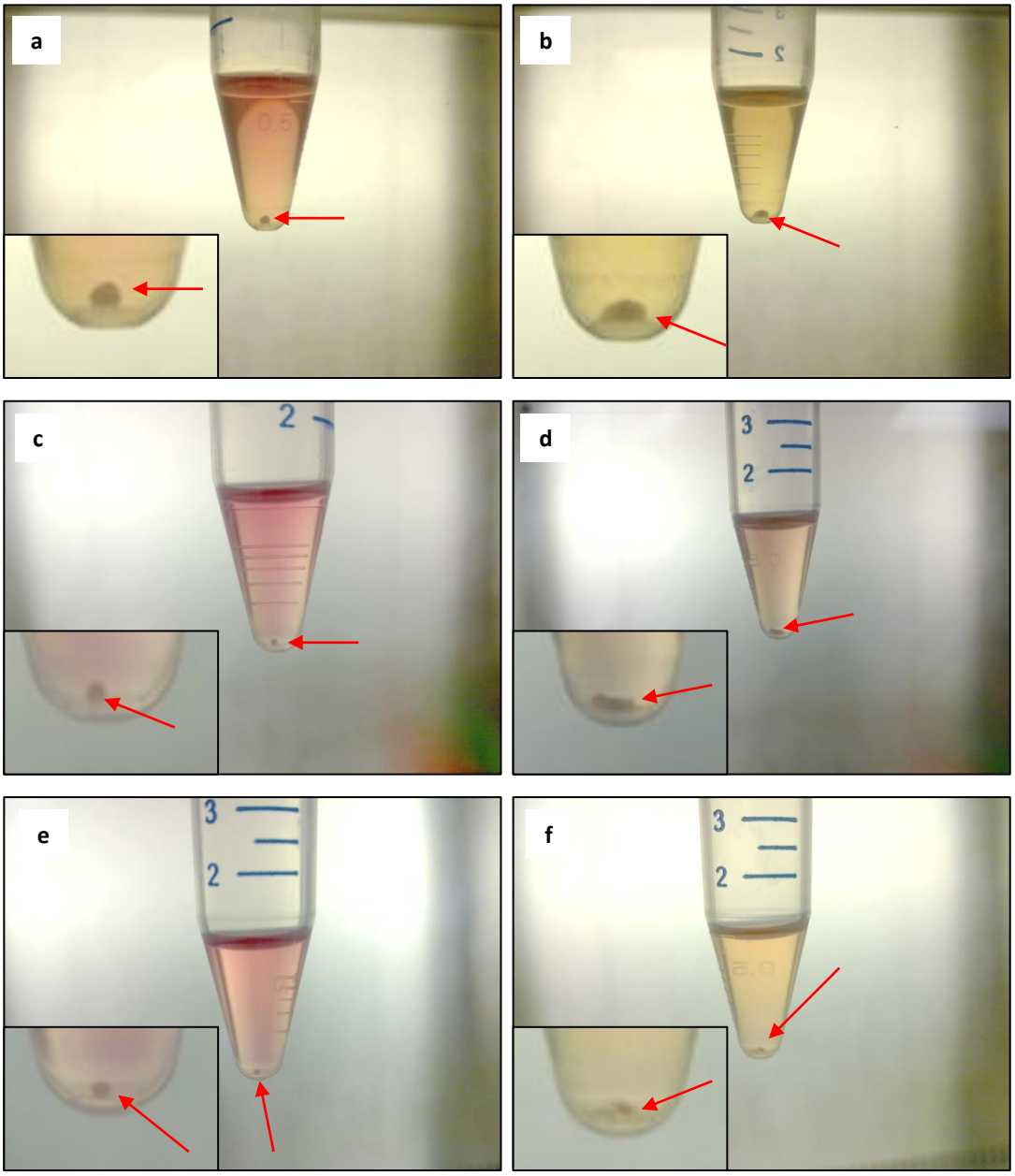



**Figure 3.5:** **Photographs of DPSC pellets cultured in ChondroDiff chondrogenic medium. After 3 days the pellet in chondrogenic medium became rounded (a), whilst the control culture remained as an irregular mass cells (b). After 15 days, the chondrogenic cultured cells formed a smaller tighter pellet (c) and the control culture remained the same (d). At 24 days, the chondrogenic cultured cells remained as a small tight pellet (e) and the control maintained as a mass of cells (f). Arrows indicate location of the cell pellets and the insets are of the ×3 magnified pellet.**

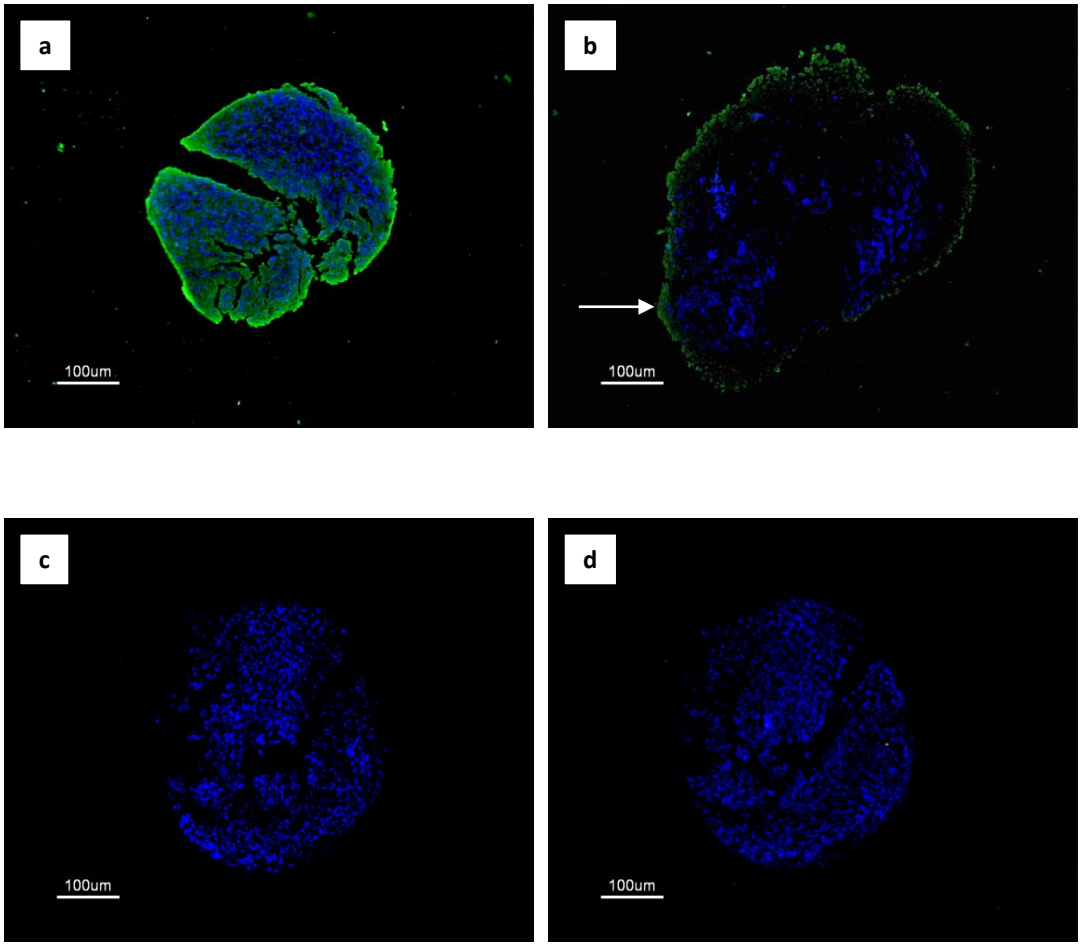



**Figure 3.6: IHC of day 24 pellet cultures. The DPSC pellets cultured in ChondoDiff chondrogenic culture appeared rounded in morphology, and were positive for cartilage 2B6 antibody for the detection of chondoitin-4-sulfate (a). The control pellet was irregular in shape with negative "edge effect" (arrowed) labelling (b). The primary exclusion control (c) and isotype control (d) samples remained negative. ×4 magnification, green fluorescence represents the anti-2B6-FITC labelling, and the blue fluorescence represents the nuclei counter-stain.**

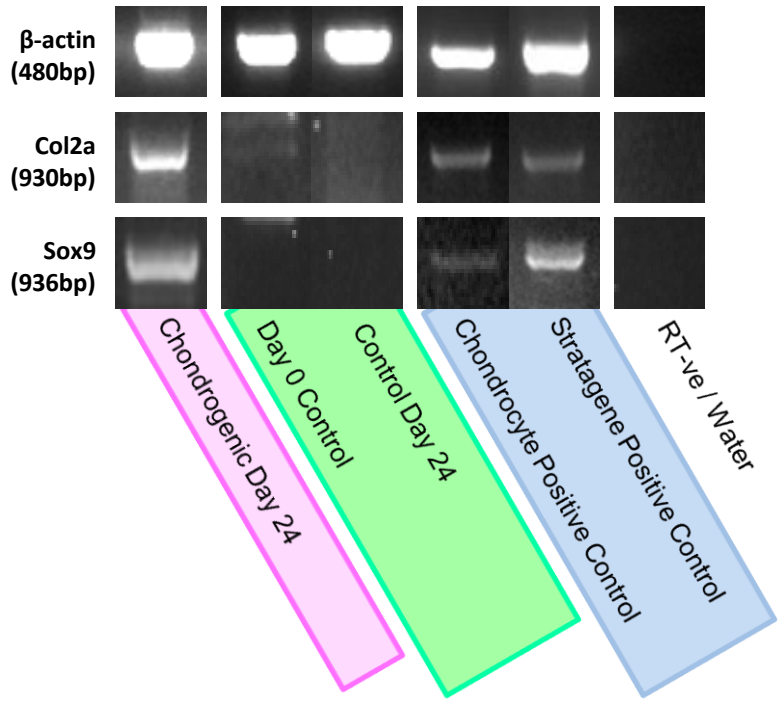



**Figure 3.7:** **Cells in chondrogenic culture expressed Col2a and sox9 chondrogenic markers, while the control cultures did not express any of the markers. The primer markers were positive with the Stratagene and chondrocyte cDNA, and all the samples were positive for β-actin housekeeping gene. The RT and water negatives remained blank.**

antigen and chondrogenic gene markers, whilst the control cultures did not demonstrate similar physical changes during culture and were negative for chondrocytic markers during IHC and RT-PCR.

### 3.3.3. Osteogenic Differentiation of DPSCs

The osteogenic cultures of DPSCs at day 12 were fibroblast-like in morphology but appeared larger and with more matrix secreted (figure 3.8a) when compared with the control cultures. The control cultures were also fibroblast-like but were narrow in morphology and the cells formed multi-layers (figure 3.8b). By day 23 of culture the osteogenic cultured cells formed multi-layers and produced clusters of matrix (figure 3.8c) whilst the control cultures remained same but with less matrix secreted and were smaller then the cells in the osteogenic cultures (figure 3.8d).

The alizarin red S staining of the osteogenic medium cultured cells were tinted pink, with secreted mineralised calcium compounds shown as globules scattered in the culture (figure 3.9a). The control cultures when stained with alizarin red S did not reveal any stained cells or mineralised matrix (figure 3.9b). ICC of the osteogenic cultured samples revealed the presence of the glycoprotein BSP found around the nuclei and at a distance from the nuclei/cells, an indication of mineralisation and osteogenesis (figure 3.10a). The control cultures did not demonstrate positive staining for BSP (figure 3.10b) and the primary antibody exclusion (figure 3.10c) and isotype controls (figure 3.10d) remained negative as experimental controls.

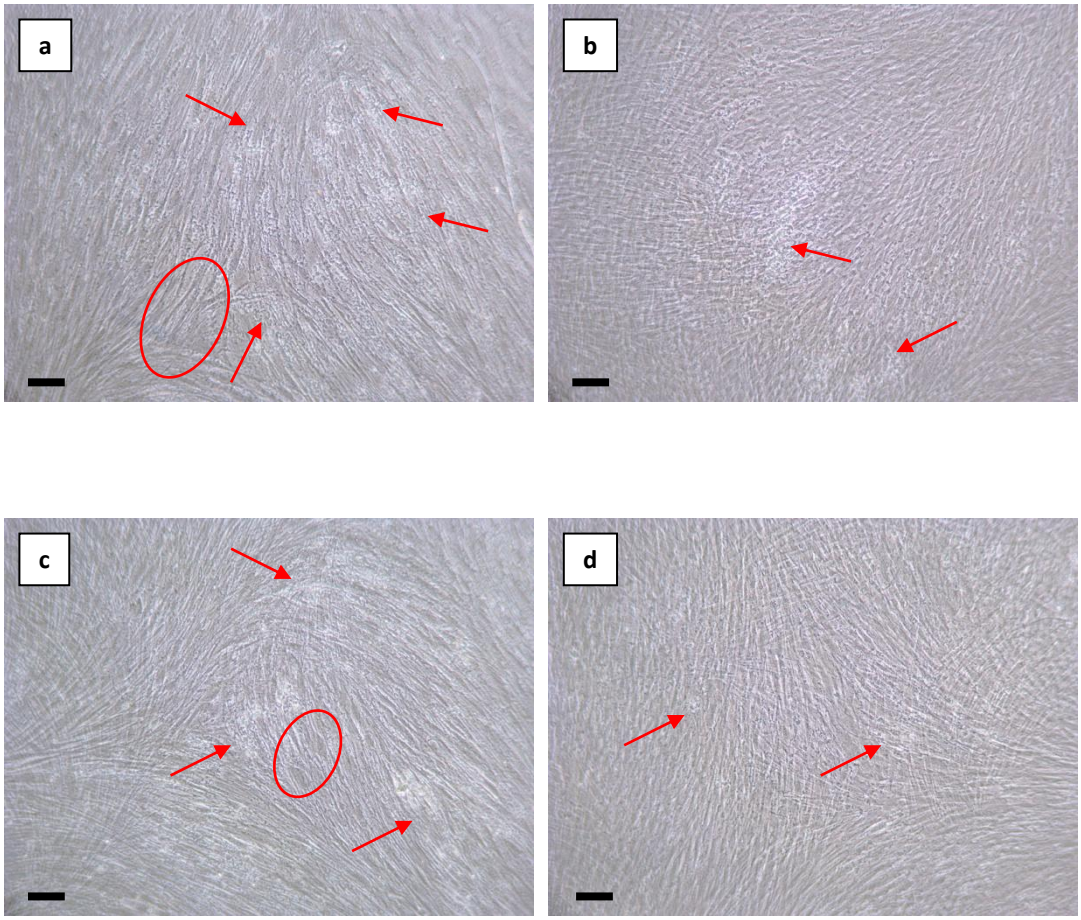
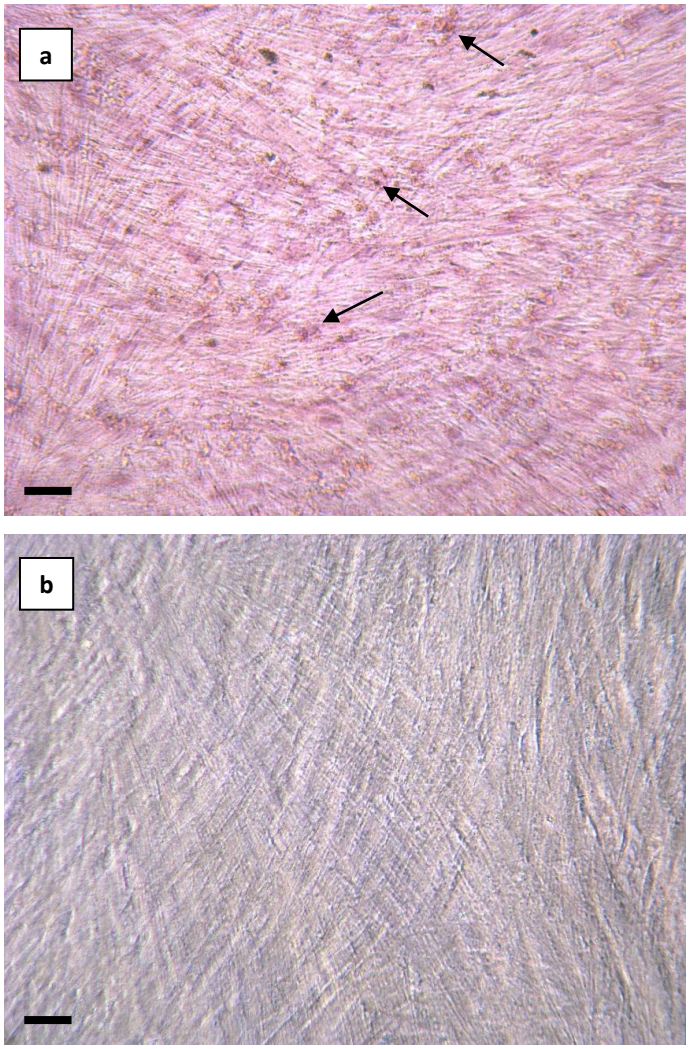



**Figure 3.8:** **Light microscopy of DPSCs cultured in osteogenic medium for 12 days appeared larger and with higher amounts of matrix secreted (a), when compared with the control cultures, which appeared fibroblast-like and narrow with overlaid cells (c). By 23 days of culture, the osteogenic cultured cells were overlaying each other and a large amount of secreted matrix was present (c), compared with the control culture which has less apparent matrix. ×10 magnification, arrows illustrate matrix secretions, circled are example large cells and scale bar represents 100 µm.**




**<u>Figure 3.9:</u> Light microscopy of osteogenic cultured cells, stained pink with alizarin red S with secreted calcium globules (arrowed) at day 23 (a). The control cultures were not stained and stained matrix was not observed (b). ×10 magnification, scale bar represents 50 µm.**

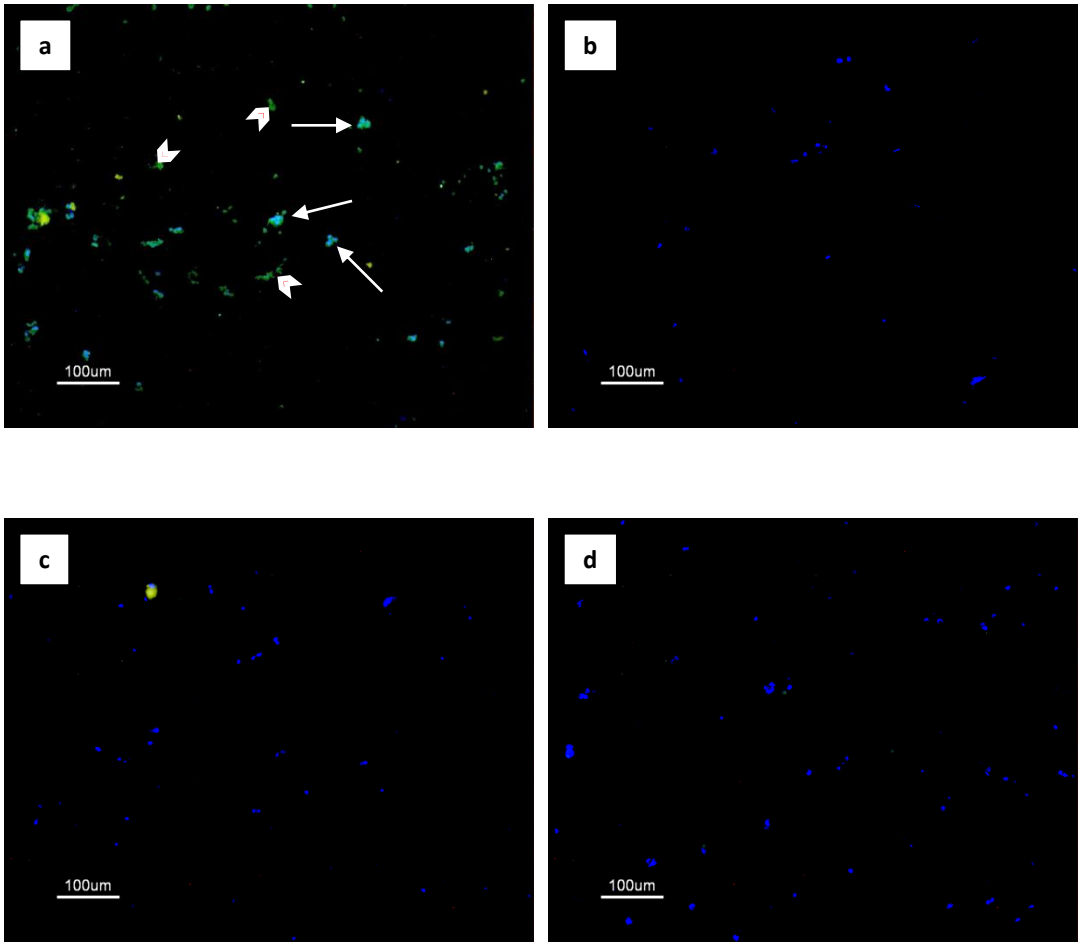



**Figure 3.10: ICC of osteogenic cultures positive for BSP around nuclei (arrowed) and at a distance from the nuclei (arrowheads) at 23 days of culture (a). The control culture was not positive for BSP (b), and the primary exclusion (c) and isotype controls (d) were also negative. ×4 magnification, green fluorescence represents anti-BSP-FITC labelling, and the blue fluorescence represents the nuclei counter-stain.**

The RT-PCR of the osteogenic cultured cells also revealed the presence of osteogenic markers OCN and BSP (figure 3.10). The day 0 initial culture controls were positive for OCN but not BSP and the day 23 control cultures were positive for both OCN and BSP. The Stratagene and osteoblast positive controls were positive for both osteogenic markers (figure 3.11). In summary the DPSCs cultured in osteogenic culture medium appeared as larger cells, secreted a large amount of matrix and expressed genes for osteogenesis. The control cultures appeared smaller, produced little matrix and also expressed osteogenic markers with long culture periods. However, the cells cultured in osteogenic medium produced BSP whilst none were detected in the control cultures.

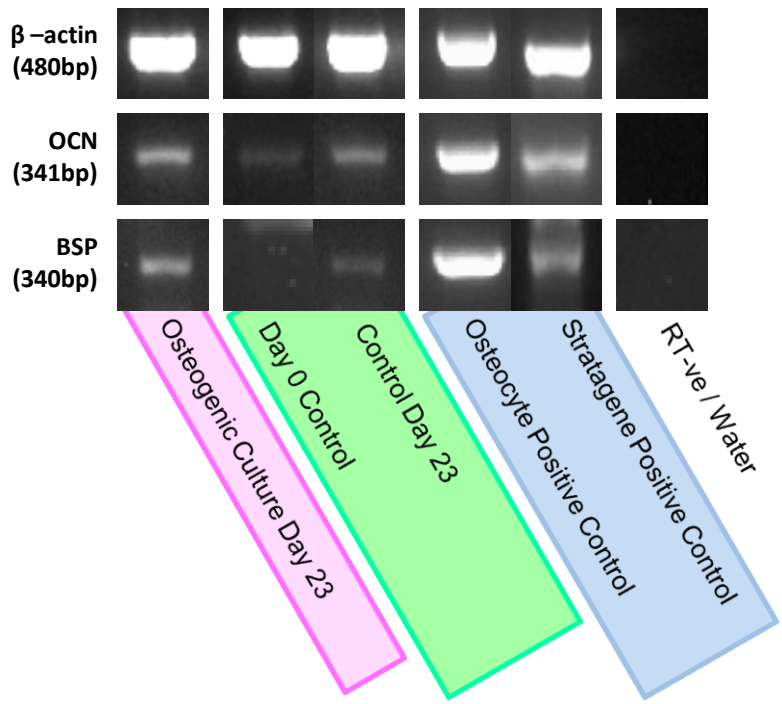



**Figure 3.11:** **Osteogenic cultured cells expressed OCN and BSP osteogenic markers, while the day 0 controls only expressed OCN and the day 23 control cultures also expressed OCN and BSP. The primer markers were positive with the Stratagene and chondrocyte cDNA, and all the samples were positive for β-actin housekeeping gene. The RT and water negatives remained blank.**

### 3.4. Discussion

Highly proliferative human DPSC clones were shown to be able to differentiate into mesenchymal lineages. The clonal cells from chapter 2 expressed multipotent MSC markers (table 2.3) and differentiated into adipogenic, osteogenic and chondrogenic lineages when cultured in the relevant induction medium. This was verified by histological and RT-PCR analysis, demonstrating multipotent characteristics of adult DPSCs.

Adult stem cell populations are valued for their proliferation and plasticity to differentiate (and possibly trans-differentiate) into multiple lineages (Mimeault and Batra 2006; Wagers and Weissman 2004). These cells are suggested to be able use as alternatives to ESCs with easier ethical accessibility, less likely to form teratomas and could be used for autogenic therapy (Stocum 2001). Well studied multipotent adult stem cells has been derived from the bone marrow, synovial, cardiac, limbal and oral tissues (Davies et al. 2010; De Bari et al. 2001; Dowthwaite et al. 2004; Ikeda et al. 2010; Stefanovic et al. 2009). This chapter utilised a clonal population of fibronectin preferentially selected human DPSCs, where the use of a clonal population has added benefits of being an "enriched" culture, unaffected by other endogenous cells present (Kotobuki et al. 2004) for the study of differentiation potentiality.

The DPSCs have demonstrated to be able to differentiate into adipogenic cells with morphological changes. The cells became engorged, lipid droplet-filled cells that expressed the lipid transcription factor PPARG2 but not lipoprotein lipase (LPL). The presence of lipid droplets is a characteristic of adipocytes and indicative of

successful differentiation of MSCs which is heavily supported in much of literature (Gronthos et al. 2000; Huang et al. 2009a; Pittenger et al. 1999). The expression of PPARG2 suggested the differentiation of DPSCs into immature adipocytes. The transcription factor PPARG2 is the most abundant isoform in human adipose tissues, its expression has been linked to play a major role in regulation of cytokine expression, apoptosis, fatty acid distribution and differentiation of progenitors into lipid-filled adipocytes (Dahlman and Arner 2007; Yoke Yin et al. 2010).

Transcription of LPL is downsteam from PPARG2 where LPL enzymatically hydolyses lipids for the release of fatty acids and is upregulated only with adipocyte maturation for the generation of localised free fatty acids (Kota et al. 2005; Rangwala and Lazar 2004; Yoke Yin et al. 2010). As LPL is expressed in mature adipocytes, its absence with the RT-PCR results suggests the differentiated adipocytes were immature and longer-term culture may develop LPL expression. Taking this into consideration the expression of early transcription factor PPARG2 and the presence of lipid droplets are an indication of successful early adipogenesis (Davies et al. 2010; Pittenger et al. 1999).

The DPSC pellets cultured in chondrogenic induction medium were remodelled into smaller condensed tissues suggestive of precartilage condensation by collagen II formation as was demonstrated in the original chondrogenesis experiment (Johnstone et al. 1998). The cell pellets cultured in chondrogenic medium probably formed new cartilage tissue as they possessed the chondoitin-4-sulfate (C4S) 2-B-6 antigen, which is normally present within mineralised tissues such as dentine and bone

matrices (Embery et al. 2001; Qin et al. 2006; Waddington et al. 2003) but also in articular cartilage (Roberts et al. 2003; Takahashi et al. 1996).

Aggregcan is the major proteoglycan in cartilage, with glycosaminoglycans (GAGs) composed of mainly chondroitin sulphate but also dermatan sulphate and keratan sulphate. It has been shown cartilage chondroitin sulphates were mainly chondroitin-6-sulfate (C6S) or C4S. Higher levels of C4S are present in lower layers of cartilage and found during the early development, and C6S in the more mature layers of cartilage (Bayliss et al. 1999; Hascall and Sajdera 1970; Ofek et al. 2008) This suggests the DPSCs differentiated into cartilage tissues, probably as immature cartilage due to the short culture period and the presence of C4S detected throughout the cell pellet. Presence of C6S was not looked at which would have been an indicator of mature cartilage.

With the expression of early transcription factor Sox9 and the major component of cartilage, type 2 collagen (Col2a), these were also indicative of cartilage formation (Shirasawa et al. 2006). RT-PCR and the histology combined provided evidence of successful differentiation of DPSCs into functional chondrocytes, which supports previous literature of chondrogenic differentiation capacity of DPSCs (Grottkau et al. 2010; Yu et al. 2010).

Culture of undifferentiated DPSCs in mineralising medium induced osteogenic differentiation. The differentiated cells demonstrated extended cytoplasmic processes and alizarin red S staining illustrated calcium-compound matrix deposits as typical mineralised globules/nodules secreted typically during DPSC mineralised tissue

formation (Gronthos et al. 2000; Ikeda et al. 2010). It was also shown that the differentiated cells secreted bone matrix marker protein BSP, known to be vital for cell interaction, mineralisation and regulation. BSP has been reported to be secreted paranuclear at the Golgi area and secreted extracellularly into multiple foci as mineralisation nucleation sites by osteoblasts (Bianco et al. 1993). The immunohistochemistry (IHC) results illustrated the differentiated DPSCs secreted BSP localised paranuclear to the cells and extracellularly from the cells.

The expression of the BSP transcription factor with RT-PCR also confirmed the positive production of BSP, indicative of matrix mineralisation when combined with the positive alizarin red S staining results. The RT-PCR also confirmed the presence of early bone marker OCN which further supports osteogenic differentiation of DPSCs. OCN has demonstrated to have a role in metabolism and is the most abundant non-collagenous protein in bone matrix expressed by pre-odontoblasts at predentine matrix secretion prior to its mineralisation (Ducy et al. 1996; Gorter de Vries et al. 1987; Kim et al. 2010; Nakashima et al. 1994). Collectively, the presence of mineralised nodules, BSP and expression of OCN confirms the osteogenic differentiation of DPSC.

Control cultures of DPSCs in culture medium expressed BSP and OCN when analysed by RT-PCR suggesting osteogenic differentiation. These did not form actual mineralised nodules nor secrete BSP but illustrated the preferential differentiation into a mineralising lineage. It was possible that the cell populations were not homogenous and had a variety of differentiated states (may be due to spontaneous differentiation) within the population. These cells may have

preferentially differentiated into a mineralised lineage by default as the cells were sourced from the teeth, which may have some directed lineage programmed already and caused positive BSP and OCN gene expression.

As the DPSCs were suggested to differentiate into odontoblast-like cells during dentine repair, the presence of dentine markers dentine phosphoprotein (DPP) and dentine sialoprotein (DSP) expressions should have been studied. The DPP is associated with calcium binding and mineral nucleation and DSP is dentine-specific, secreted by immature odontoblasts at the predentine front (Bronckers et al. 1994; D'Souza et al. 1992). The presence of these markers would further clarify if the mineralised cells differentiated into odontoblast-like cells or not since BSP and OCN are both mineralisation markers present for both osteoblast and odontoblast cell types.

This chapter has established the multipotent differential characteristics of a clonal FNA DPSC population *in vitro*, which supports previous studies where dental derived DPSCs can differentiate into mesenchymal lineages (Gronthos et al. 2002; Shi et al. 2005; Waddington et al. 2009; Yu et al. 2010). The use of a homogenous clonal population of DPSCs and its differentiation into all three adipogenic, chondrogenic and osteogenic lineages has not been previously demonstrated, where previous groups have either differentiated DPSCs into a single lineage (typically into osteogenic or odontogenic lineages) or utilised the whole heterogeneous pulp population of progenitors, which is known to contain multiple progenitors of variable differential states which may have implications for experimental reproducibility.

It has been previously shown that the multitude of progenitors within the dental pulp are multipotent, the SHED populations were demonstrated to differentiate into osteogenic, odontogenic, adipogenic and neurogenic lineages (Huang et al. 2009b; Miura et al. 2003; Shi et al. 2005), immature papillae cells (SCAPs) formed adipogenic, odontogenic and osteogenic lineages (Lei et al. 2011; Sonoyama et al. 2008) and PDL progenitors (PDLSCs) differentiated into osteogenic, adipogenic and chondrogenic lineages (Fujii et al. 2008; Gay et al. 2007; Ikeda et al. 2010), making the dental pulp a valuable source of progenitors that may be utilised for tissue engineering experiments.

The use of a homogenous multipotent clonal population may have a role for tissue engineering and developing novel therapeutic applications, with the use a patient-matched source of progenitor cells for regenerative therapy. The establishment of this multipotent DPSC clone allows for reproducible study of signalling bioactive molecules released during dentine injury and its effects on DPSC behaviour.

# Chapter 4:

# Effects of Dentine Matrix Proteins on Human Dental Pulp Stem Cell Viability, Proliferation and Migration

## 4.1. Introduction

The maintenance and repair of body tissue requires the delicate cell-cell and cell-matrix interactions within its local microenvironment (Discher et al. 2009; Fuchs et al. 2004; Li and Xie 2005). This "niche" has an intricate role over stem cell fate with contributing factors consisting of mechanical forces, matrices, growth factors and cytokines. These factors, individually and collectively, act as autocrine or paracrine effectors governing stem cell migration, survival, proliferation and its differentiation (Discher et al. 2009; Sloan and Waddington 2009).

The dentine extracellular matrix (ECM) is secreted by differentiated odontoblasts (Linde and Goldberg 1993), the dentine matrix proteins (DMPs) are released during dentine injury by solubilisation with pathogenic acids, host proteolytic enzymes (Dung et al. 1995) and restorations using calcium hydroxide ($Ca(OH)_2$) or ethylenediaminetetraacetic acid (EDTA) (Goldberg and Smith 2004; Graham et al. 2006; Javelet et al. 1985; Kardos et al. 1998). The DMP includes collagenous and non-collagenous proteins (NCPs) such as proteoglycans, matrix proteins and growth factors, all of which play a role in regulating mineralisation during dentine development and repair.

Previous experiments that implanted BSP into exposed pulps of rat teeth have demonstrated a reduction in inflammation after 7 days and formation of new dentine

after 14 days, illustrating its regenerative properties (Goldberg et al. 2009). However, studies utilising single bioactive molecules for dentine repair may not be an accurate depiction of *in vivo* repair. The dentine matrix contains a "cocktail" of bioactive molecules which includes TGF-β1, VEGF and IGF amongst NCPs that allow complex/synergistic interactions that may modulate and regulate many processes. These processes include DPSC proliferation, migration and differentiation, vital as part of dentine repair and development (Cooper et al. 2010; Goldberg et al. 2009; Goldberg and Smith 2004). This complexity suggests the use of whole DMP extracts to investigate dentine repair is a more valid approach for assisting development of novel therapeutic strategies.

Early studies that utilised DMP for dentine repair demonstrated a reactionary and reparative dentinogenesis response *in vivo* (Smith et al. 1995; Smith et al. 1994). The commonly used $CaOH_2$ during cavity preparations has been shown to induce dentine regeneration by releasing the local matrix proteins (Graham et al. 2006). The use of EDTA-extracted DMP were shown to initiate a reactionary/reparative response (Smith et al. 1994; Smith et al. 1990) where the use of EDTA-DMP to line deep cavities of primate teeth demonstrated greater reactionary dentine deposition compared with using a $CaOH_2$ cement, although both produced a greater reactionary dentinogenesis compared with resin-modified glass ionomers (Duque et al. 2006). Both experiments demonstrated an enhanced dentine repair with the use of DMP.

Much of literature has shown a reactionary/reparative dentinogenesis response achieved by the addition of whole DMP fractions, however no studies has shown its specific effects on the DPSCs behaviour, whether the matrix proteins cause an

increase or decrease to DPSC viability, proliferation or migration and if there is a dose-dependant response.

This chapter aims to further understand cellular events that occur during dentine regeneration by use of EDTA-extracted DMP and investigating the effects it has when applied to a clonal population of DPSCs. Specifically, the effect of DMP on cell proliferation will be ascertained by cell counts, DMP effects on cell vitality will be would be determined by colourimetric/luminescence assays in conjunction with RT-PCR for apoptotic markers. Any migratory responses caused by DMP will be assessed with use of Boyden chambers and cell counting. These results would provide insight to DPSC behaviour during dentine injury and when DMP is used during pulp-capping experiments, but also identifies if DMP has any detrimental effects to DPSCs that may make it clinically unsuitable as a pulp-capping component.

## 4.2 Materials and Methods

### 4.2.1. Cell Source

The isolated DPSC clonal populations hFNA3 clone C4 (24 PDs) and hNA clone F7 (23 PDs) were used. Both populations expressed multipotent and pluripotent gene markers (section 2.3.6) where hFNA was also shown to be multipotent (section 3.3).

### 4.2.2. Culture Medium

Culture medium was composed of alpha-modification Minimum Essential Medium (αMEM) pre-supplemented with ribonucleosides and deoxyribonucleosides (Gibco, Invitrogen, UK), further supplemented with hi-FCS, L-glutamine, L-ascorbate 2-phosphate and antibiotics/antimycotics as previously described (section 2.2.2).

### 4.2.3. Source of Dentine Matrix Proteins

Lyphophilised human dentine matrix proteins (DMPs) were previously prepared by removal of the enamel and cementum layers, powdered under liquid nitrogen, then solubilised in 10% EDTA (pH 7.2) (Baker et al. 2009; Tomson et al. 2007). The adult teeth were obtained following informed patient consent (Oral Surgery Department, Birmingham Dental Hospital, UK) and the DMP was provided for use in this chapter as a lypholised powder by Dr AJ Sloan (Cardiff University, UK). The powdered DMP was resuspended in αMEM as 1 μg/μL stock concentration and stored at -20℃. The protein profile has been verified by another member of the research group by Western blotting and silver staining which confirmed presence of proteins which include TGF-β1 (Sadaghiani et al. 2010).

### 4.2.4. Generating a Negative Dentine Matrix Protein Control ("DMP-Neg")

Removal/denaturing of collagenous and NCPs of the DMP were performed to create a negative control ("DMP-Neg"). This was completed by treating 100 µg DMP powder with 1 mL of 4 M guanidine chloride (GuCl; Fisher Scientific, UK) and 0.05 M sodium acetate (Sigma-Aldrich, UK) at pH 6.8 in a sterile 1.5mL eppendorf (Eppendorf, UK) with agitation at 4 ℃ for 48 hrs. This was then centrifuged at 500 ×g for 10 mins and the NCP supernatant was carefully aspirated and discarded. The pellet was washed with 1 mL dd.$H_2O$ and centrifuged at 500 ×g for 10 mins to ensure removal of all NCPs. The remaining collagenous protein was resuspended in dd.$H_2O$, transferred to a 1 cm wide cellulose dialysis tube (Sigma-Aldrich, UK) for dialysis against dd.$H_2O$ for 48 hrs at 4 ℃.

The dialysed product was transferred to a 1.5 mL eppendorf, frozen at -20 ℃ and lyphophilised in a -50 ℃ freeze drier (Edwards Modulyo, UK). For the removal of collagenous protein the lyophilised product was digested with 100 µL pre-warmed 4 mg/mL collagenase/dispase (Roche Applied Science, UK) at 37 ℃ for 1 hr with regular agitation. This was then exhaustively dialysed against dd.$H_2O$ for 48 hrs at 4 ℃ and the de-proteinated DMP ("DMP-Neg") was lyphophilsed and resuspended with 100 µL αMEM for a final concentration of 1 µg/µL for storage at -20 ℃.

### 4.2.5. Activation of TGF-β1

Recombinant human TGF-β1 derived from HEK293 cells was supplied as 5 µg lyphophilised powder (PeproTech, USA). TGF- β1 was activated by the addition of 100 µL 10 mM pH 3 citric acid (Fisher Scientific, UK) with 400 µL of 0.1% BSA-

PBS (BSA from Fisher Scientific, UK) as a final concentration of 10 μg/mL TGF-β1 and stored at -20 ℃.

### 4.2.6. Cell Assay Standard Growth Curve

A standard cell seeding density for use in 96-well plate experiments was determined using a colourimetric MTS assay. In two 96-well plates (Greiner Bio-One, UK), DPSCs were cultured in 200 μL culture medium per well with $2\times10^4$ cells and serially diluted 1:2 to 0 cells. Each concentration was cultured (with repeats) as triplicate wells in the plate at 37 ℃, 5% $CO_2$ and changing with fresh medium every 48 hrs. At 72 and 120 hrs of culture the media from each of the wells were aspirated with 100 μL of fresh culture medium and 20 μL MTS assay added (CellTiter 96® $AQ_{ueous}$ One Solution Cell Proliferation Assay, Promega, UK). This was incubated at 37 ℃ for 2 hrs in darkness before taking a 544 nm absorbance reading on a 96-well plate reader (FLUOstar OPTIMA, BMG Labtech, Germany).

Absorbances from the sample wells were standardised against the absorbance of wells containing cells without MTS reagent minus absorbance from wells with cells but no MTS reagent. This produced a standard curve which determined $5\times10^3$ cells/well is a suitable density remaining in log phase of growth at 72 and 120 hrs of culture.

### 4.2.7. Determining DPSC Viability with MTS Assay

Cell proliferation was quantified using an MTS assay (section 4.2.6) for determining amount of viable cells. The DPSCs (n = 2, at 23 and 27 PDs) at $5\times10^3$ cells/200 μL culture medium were cultured overnight at 37 ℃ and 5% $CO_2$ in each well of a 96-

well plate (Greiner Bio-One). The medium was aspirated and replaced with 200 µL culture medium treated with 10 µg/mL DMP with a 1:2 serial dilution to 0 µg/mL. For the negative control, DMP-Neg was used instead of DMP at the same serial dilution. As a positive control, TGF-β1 was used at 100 ng/mL and serially diluted 1:2 to 0.78 ng/mL with a final well at 0 µg/mL as a control. These were performed in triplicate wells (with repeats) and were cultured with medium change at 48 hrs. After 24, 48 and 72 hrs of culture the medium was replaced with 100 µL of fresh culture medium and 20 µL MTS assay for taking an absorbance reading which was standardised with the control wells as described previously (section 4.2.6) and used for comparisons.

### 4.2.8. Determining DPSC Number Expansion with Crystal Violet

A measure of cell numbers was determined with crystal violet staining. DPSCs were cultured in the same set-up in 96-well plates as described previously (section 4.2.6). After 24, 48 and 72 hrs of culture the media was aspirated and the cells fixed overnight at 4℃ in ice-cold 70% ethanol (Sigma-Aldrich, UK). The ethanol was replaced with a mixture of 2% ethanol with 0.1% crystal violet powder (BDH Laboratory Supplies, UK) in 0.1 M Borax solution (Sigma-Aldrich, UK) at a final pH 9. Samples were incubated at room temperature on a rocker for 1 hr (or until cells were visibly stained purple) then gently rinsed with cold running water until the water ran clear. The cells were then air dried before viewing under light microscopy for counting and comparison

### 4.2.9. Quantification of Apoptotic Activity

The Caspase-Glo 3/7 Assay (Promega, UK) reagent was prepared by thawing and adding the entire 10 mL Caspase-Glo 3/7 Buffer to the lyphophilised Caspase-Glo 3/7 Substrate (when using 10 mL size kit, Promega, UK) and gently swirled to mix the reagents. This was either used immediately or stored (4 ℃ or -20 ℃) to be used within 4 weeks, warming to room temperature before use. DPSCs were cultured in black (not optically clear) 96-well plates (Greiner Bio-One) in the same layout as described previously (section 4.2.6). After 24, 48 and 72 hrs of culture the medium was replaced with 25 µL of culture medium and 25 µL of Caspase-Glo 3/7 reagent. The 96-well plate was mixed for 30 s on a plate shaker and incubated at room temperature for 2 hrs. The plate was then transferred to a plate reader (FLUOstar OPTIMA, BMG Labtech, Germany) at maximum optical gain settings (maximum sensitivity to light) to measure the luminescence as arbitrary relative luminescence units (RLUs) of each well, which was used for quantitative comparison of each well.

### 4.2.10. Determination of Apoptotic Marker Expression

DPSCs were seeded in triplicates at $2 \times 10^3$ cells/well into 6-well plates (Greiner Bio-One) with 2 mL of culture medium/well and cultured overnight at 37 ℃, 5% $CO_2$. The culture media was replaced with 2 mL DMP supplemented medium at 10 µg/mL, 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL or 0.001 µg/mL with 0 µg/mL as control. DMP-Neg was also used at the same 10 µg/mL, 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL, 0.001 µg/mL and 0 µg/mL, with TGF-β1 as a positive control at 100 ng/mL, 10 ng/mL and 1 ng/mL with 0 µg/mL control. These were performed in triplicate wells, and cultured with fresh media changes at 48 hrs.

After 24, 72 and 120 hrs of culture the cells were washed with PBS buffer before lysis with Buffer RLT (Qiagen, UK) with 10 µL/mL of β-mercaptoethanol (Sigma-Aldrich, UK) for RNA extraction, quantification, RT-PCR and agarose gel analysis (as described previously from section 2.2.13.1 to 2.2.13.6.). Primers used were human apoptotic, survival and proliferation markers with β-actin as the housekeeping gene (table 4.1)

**Table 4.1: Apoptosis, survival and proliferation markers for PCR amplification. β-actin was used as a housekeeping gene.**

| ***Gene Product*** | **Human** | | | |
|---|---|---|---|---|
| | ***Primer Sequence 5' - 3'*** | ***Ta (°C)*** | ***Product Size (bp)*** | ***Source*** |
| Caspase3 *casp3* | Fwd: 5'-GTTTGTGTGCTTCTGAGCCA-3'<br>Rev: 5'-TCAAGCTTGTCGGCATACTG-3' | 55 | 350 | Author's |
| Bax *bax* | Fwd: 5'-GGGGACGAACTGGACAGTAA-3'<br>Rev: 5'-CCCAAAGTAGGAGAGGAGGC-3' | 55 | 300 | Author's |
| Akt1 *akt1* | Fwd: 5'-GTGCCACCATGAAGACCTTT-3'<br>Rev: 5'-CATCTTGGTCAGGTGGTGTG-3' | 55 | 459 | Author's |
| PCNA *pcna* | Fwd: 5'-GGCGTGAACCTCACCAGTAT-3'<br>Rev: 5'-CTTCATCCTCGATCTTGGGA-3' | 55 | 571 | Author's |
| β-actin *actb* | Fwd: 5'-AGGGCAGTGATCTCCTTCTGCATCCT-3'<br>Rev: 5'-CCACACTGTGCCCATCTACGAGGGGT-3' | 65 | 480 | Dr. XQ Wei (Cardiff University) |

### 4.2.11. Migration of DPSCs in Presence of Dentine Matrix Proteins

Liquid rat tail collagen I at 3.91 mg/mL in 0.02N acetic acid (BD Biosciences, Scientific Laboratory Supplies, UK) was formed into 1 mg/mL gels/lattices using culture medium and acid neutralisation with sterile 1N sodium hydroxide (NaOH) (Fisher Scientific, UK).

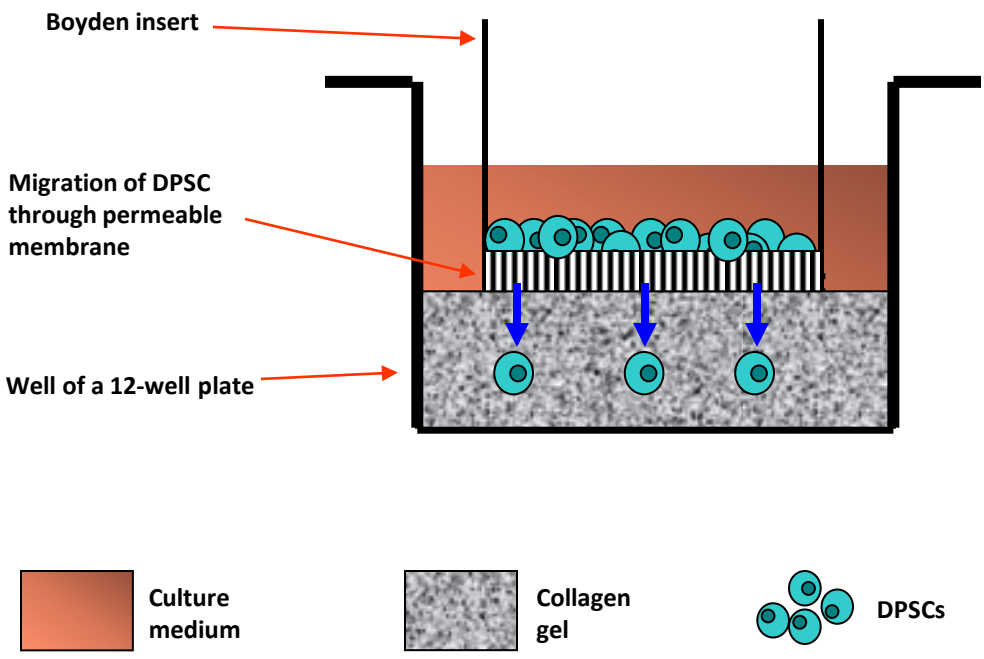
For 7 mL of collagen gel, 5.169 mL culture medium was added to 1.79 mL collagen stock, mixed gently to avoid bubbles, before the acetic acid neutralised with 41 µL of

1N NaOH and gently mixed (avoiding bubbles), to be immediately transferred as 1 mL/well into 12-well tissue culture plates (Greiner Bio-One, UK) to solidify overnight at 37°C. The volumes of culture medium and NaOH used is variable, dependant on collagen batch concentrations (ranging from 3-4 mg/mL), and the addition of DMP/DMP-Neg/TGF-β1 supplements were displaced from the volume of culture medium.

Tissue culture inserts with 8 μm pore membranes (Greiner Bio-One) were placed into the wells in contact with the collagen gels, avoiding air bubbles between the surfaces. DPSCs were seeded at $1\times10^4$ /cm$^2$ in 600 μL culture medium into each insert. The addition of 400 μL of culture medium to the external side of the inserts was used to balance the media volumes inside of the wells. DMP and DMP-Neg were used at 10, 1 and 0 μg/mL in the collagen gels, and TGF-β1 at 100, 10, 1 and 0 ng/mL concentrations. A simple schematic of the Boyden chamber set-up is shown in figure 4.1. The Boyden chamber assays were then cultured at 37℃, 5% $CO_2$ with fresh medium change at 48 hrs. At 24 and 72 hrs of culture, the inserts were discarded and medium aspirated to gently wash the gels twice with 1 mL/well PBS buffer. The cells in the collagen gels were fixed with 1 mL/well 4% paraformaldehyde (PFA) at room temperature for 30 mins. The gels were washed twice with 1 mL/well PBS and then left at 4℃ in 1 mL PBS until required.

### 4.2.12. Fluorescence Staining of Cells within Collagen Gels

Any cells present in the collagen gels were permeated with 500 μL/well 0.3% triton-X 100 (Boehringer Mannheim, Germany) for 30 mins with gentle agitation at room temperature. The gels were then washed 3× 1 mL/well TBS (pH7.5) for 10 mins and




**<u>Figure 4.1:</u> Schematic of a Boyden chamber. The DPSCs in an insert that has a permeable membrane, resting on a collagen gel and submerged in culture medium.**

then blocked with 500 µL/well of 1% BSA-TBS for 30 mins at room temperature with gentle agitation. The gels were stained with 500 µL/well 20 µg/mL phalloidin-FITC (Sigma-Aldrich, UK) in 1% BSA-TBS and incubated in darkness at 4℃ for 1 hr with gentle agitation. The gels were then washed 3× with 1 mL/well TBS for 10 mins before nuclei counter-stain bisBenzimide (Sigma-Aldrich, UK) was added 500 µL/well at 2.5 µg/mL diluted in 1% BSA-TBS. This was incubated in the dark for 30 mins at 4°C with regular agitation, before the gels washed 3× 1 mL/well TBS and stored overnight in TBS at 4℃ in darkness. For imaging, the gels were carefully lifted from the wells using a small spatula and transferred onto a glass slide to view with an UV microscope (section 2.2.8) at 373 nm/456 nm (FITC) and 490 nm/520 nm (bisBenzimide).

Gels were also examined by confocal microscopy (Leica TCS SP2 AOBS spectral confocal microscope, Leica Microsystems, UK) with laser excitation used at 488 nm (FITC) and 405 nm (bisBenzimide/DAPI). Z-axis image sections were captured at 10 µm intervals from the top-most cell as an arbitrary starting point before the images were manipulated into a 3-dimensional (3D) image using the provided Leica Confocal Software.

### 4.2.13 Automated Cell Counting For Semi-Quantification

Cells nuclei counter-stained with bisBenzimide (section 4.2.12.) were automatically counted using computer software ImagePro Plus (Media Cybernetics, Inc., USA). The software was calibrated to count cell nuclei in the captured images, ignoring larger or smaller clusters of pixels that were not nuclei. Each cell nuclei was assumed to be a representation of a single cell (which was verified when transposed onto the

FITC images of the cell cytoskeleton). Using 5 random fields of view at 10× magnification to generate 5 images for each condition (DMP, TGF-β1 and DMP-Neg), the cell numbers were averaged to produce semi-quantitative count for comparison with unsupplemented cultures.

### 4.2.14. Statistical Analysis

The average values and standard error of mean (SEM represented as ±) were determined using computer statistical software GraphPad Instat (USA). A post-hoc Tukey-Kramer multiple comparisons test was performed if analysis of variance

## 4.3. Results

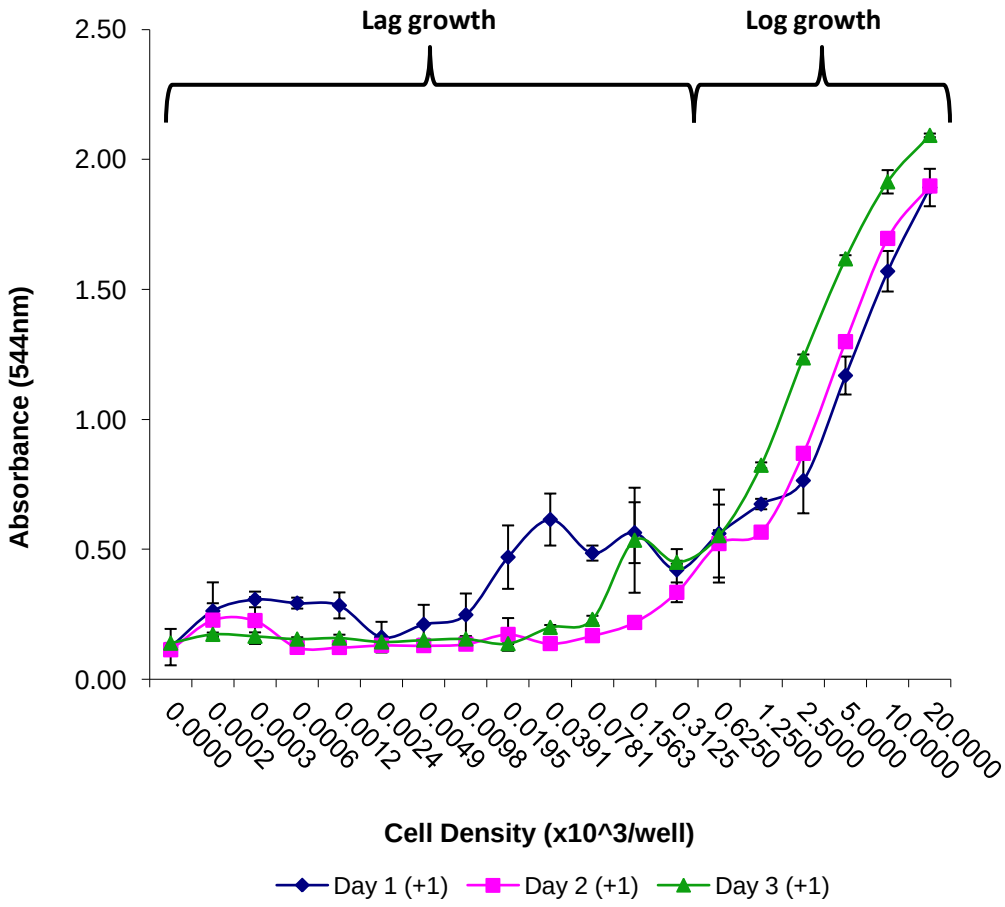
### 4.3.1. DMP Influence on DPSC Viability and Cell Numbers

Optimal cell density in log phase growth was determined from standard growth curve using hFNA (clone C4) cells at 24 PDs (figure 4.2). A density of $5 \times 10^3$ cells/well was chosen where the cells demonstrated a log increase in cell viability with culturing.

The addition of DMP to the hFNA DPSCs did not alter the MTS assay cell viability even with 72 hrs in culture. After 24 hrs the addition of DMP did not cause significant changes ($P > 0.05$) in cell vitality even with up to 10 µg/mL DMP compared with the unsupplemented control cultures (figure 4.3a). There were similar results at 48 hrs and 72 hrs with no significant difference between the control wells and DMP supplemented wells, although the increase in absorbance shown by the control wells from 24, 48 and 72 hrs (0.84, 0.91 and 0.98 respectively) indicated an increase of cell numbers with culture time but not due to supplementing with DMP.

The addition of TGF-β1 caused a significant increase ($P < 0.05$) in cell vitality compared with the control wells, with the effects of lower concentrations of TGF-β1 detectable with longer culture (figure 4.3b). Similar in results to the addition of DMP the addition of DMP-Neg did not cause significant changes in DPSC vitality (figure 4.3c).

As a measure of DMP effect on cell expansion, cultured cells supplemented with DMP were stained with crystal violet (figure 4.4a). A longer period of culture with DMP produced an increased cell count and the higher concentration of DMP also




**<u>Figure 4.2:</u> Standard curve of hFNA clone C4 (24 PDs) cultured 24hr for cell adherence, then further 24, 48 or 72 hrs before measuring cell proliferation with MTS assay (n = 6). Cell densities demonstrating lag or log phase growth at the timepoints are illustrated. Error bars are SEM.**

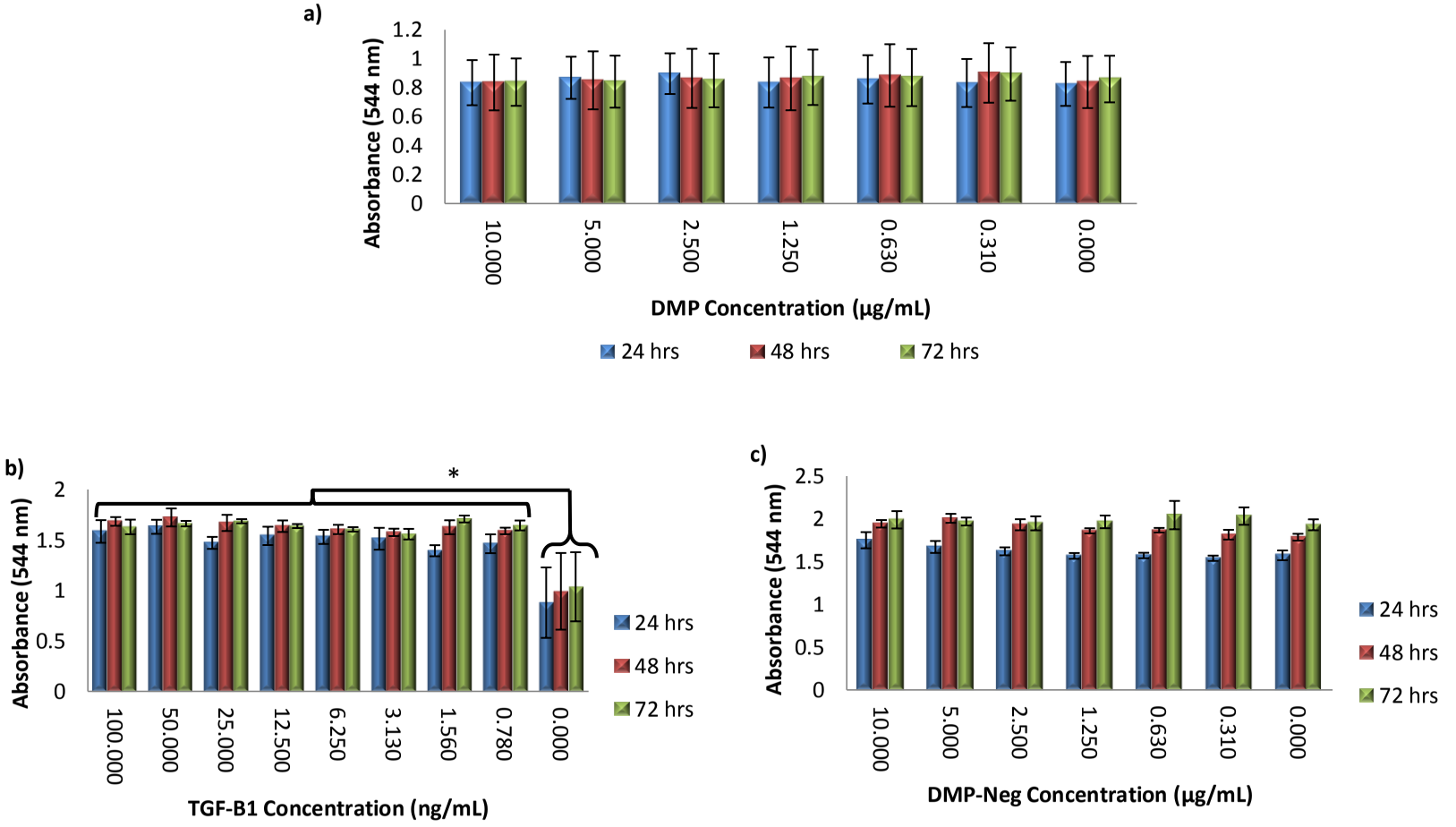



**Figure 4.3:** MTS assay for hFNA cell vitality at 24, 48 and 72 hrs (n = 6) when cultured supplemented with DMP (a), TGF-β1 positive control (b), or DMP-Neg negative control (c). Results are compared with the unsupplemented control cultures. Bars represent SEM and * = $p<0.05$.

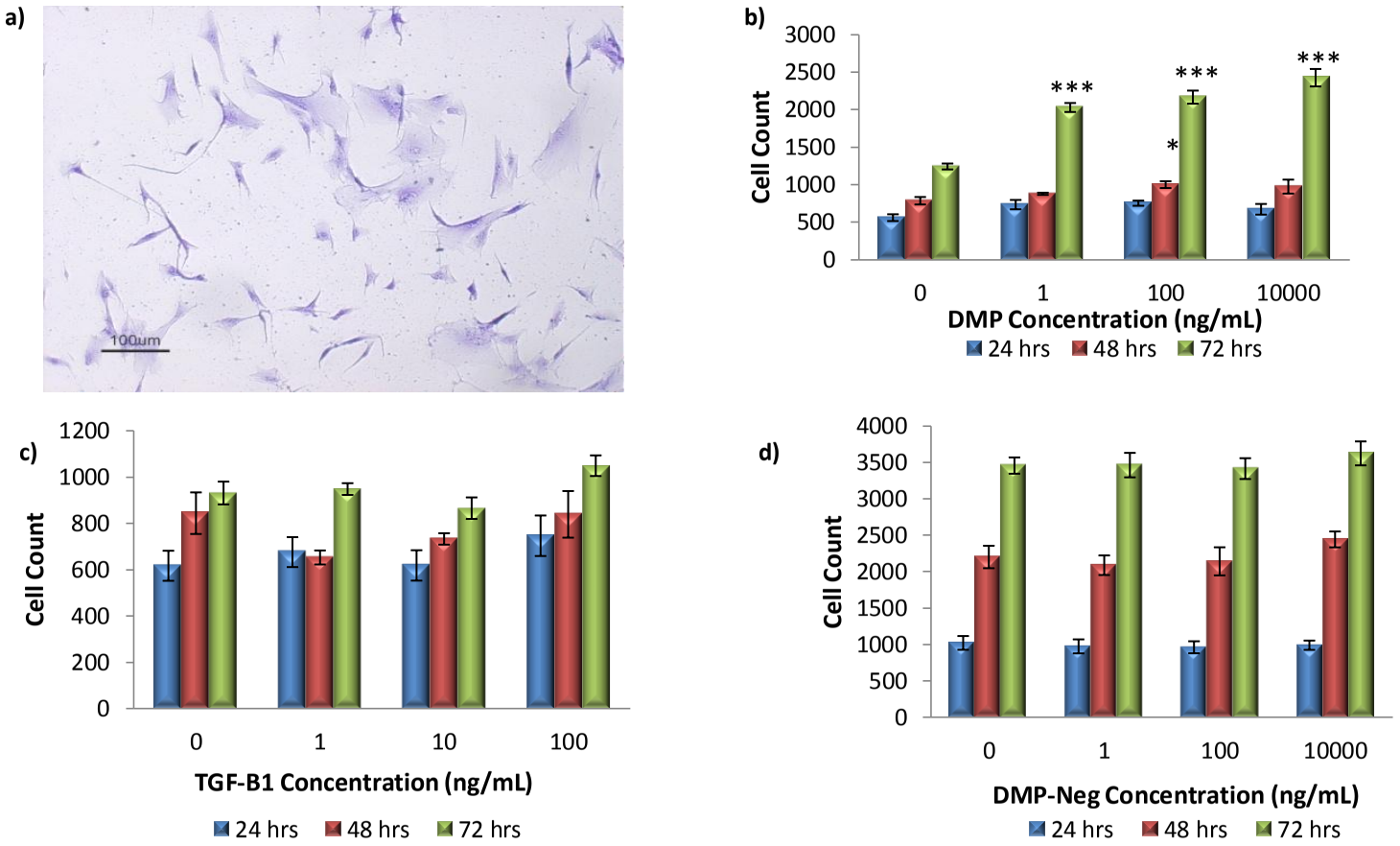



**Figure 4.4:** hFNA cells stained with crystal violet (a), after culturing for 24, 48 or 72 hrs with supplemented medium (n = 6). Cells were supplemented with DMP (b), TGF-β1 (c), or DMP-Neg (d) before stained for cell counts. Cell counts were compared with the unsupplemented controls for each timepoint. Image is ×10 magnification, bars represent SEM and * = P<0.05, *** = P<0.001.

increased cell numbers. With 24 hrs of culture no significant difference was observed between the unsupplemented control counts and cultures supplemented with up to 10,000 ng/mL DMP (figure 4.4b). At 48 hrs there were significant differences in cell counts between the control cultures and the 100 ng/mL DMP supplemented cultures. A highly significant increase ($P < 0.001$) in cell numbers was demonstrated at 72 hrs of culture between the control counts and the addition of DMP where 1 ng/mL DMP had almost double the cell count of the control. Increasing the DMP concentration showed an increase in cell count with a significant count increase from 1 ng/mL to 10,000 ng/mL DMP cultures.

The addition of TGF-β1 caused no significant changes with cell numbers when stained with crystal violet. The unsupplemented control culture illustrated a significant increase in cell numbers from 24 hrs to 72 hrs of culturing, demonstrating the cells undergo normal cell expansion but were not affected by supplementing with TGF-β1 (figure 4.4c). A similar result is shown with the addition of DMP-Neg, the control cultures show a highly significant increase in cell numbers from 24 hrs to 72 hrs of culturing but the addition of DMP-Neg causes no statistically significant differences to cell numbers (figure 4.4d). In summary the increase in cell counts with culture time indicated normal cell expansion but the addition of TGF-β1 or DMP-Neg had no effect on cell numbers compared with the effects of DMP which increased cell numbers.

### 4.3.2. Effect of DMP on DPSC Apoptosis

Culturing the hFNA cells with DMP reduced apoptotic caspase activity. Supplementing DPSCs with higher concentrations of DMP at 5 and 10 µg/mL

reduced caspase activity significantly compared with the controls especially for the 24 and 72 hr cultures. The lower concentrations of DMP between 0.01 to 2.5 μg/mL appeared not to cause any significant changes to caspase activity when compared with the unsupplemented control cultures, although appeared to have higher activity (figure 4.5a). TGF-β1 (figure 4.5b) or DMP-Neg (figure 4.5c) supplemented cells had demonstrated no discernable effect on caspase activity, with the majority of the supplemented cultures having similar relative luminescent units (RLUs) to its controls.

In support of the caspase assay, mRNA expression of apoptotic markers caspase 3 and Bax were determined along with survival marker Akt1 and proliferation marker PCNA. The mesenchymal markers CD105, CD146 and Msx-1 were investigated if they remain present after culturing in DMP, TGF-β1 or DMP-Neg. Culture of the hFNA cells for up to 120 hrs (5 days) in DMP-medium revealed the cells had maintained a continuous expression of both apoptotic and survival/proliferation markers with constant expression of mesenchymal markers even with supplementation with up to 10 μg/mL of DMP (figure 4.6 and summarised in table 4.2).

Cells expressed both apoptotic and survival markers even when supplemented with up to 100 ng/mL TGF-β1 for 24 hrs. After 72 hrs there was a discrepancy in apoptotic marker expression, with either Caspase3 or Bax expressed at the different concentrations of TGF-β1. The survival marker Akt1 was only found at 1 ng/mL TGF-β1 and even not present in the 0 ng/mL controls, although the proliferation marker PCNA was expressed where survival marker Akt1 was absent. By 120 hrs of

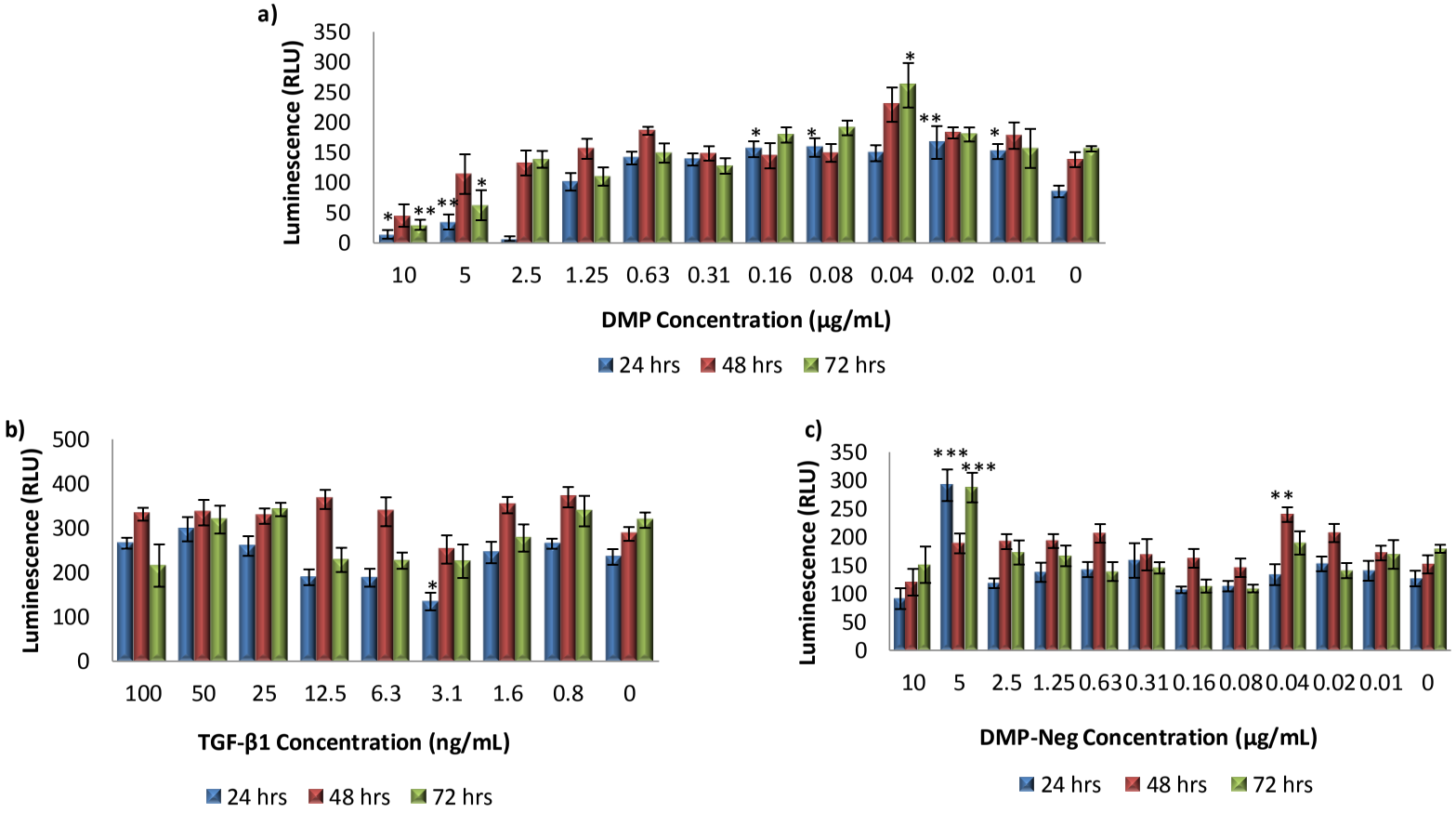



**Figure 4.5:** **Apoptotic caspase activity of hFNA cells (n = 6) supplemented with DMP (a), TGF-β1 (b) or DMP-Neg (c). Cells were cultured for 24, 48 and 72 hrs with caspase activity compared with base-level caspase activity of unsupplemented controls. Caspase activity measured as relative luminescence units (RLUs), bars represent SEM and * = P<0.05, ** = P<0.01 and *** = P< 0.001.**

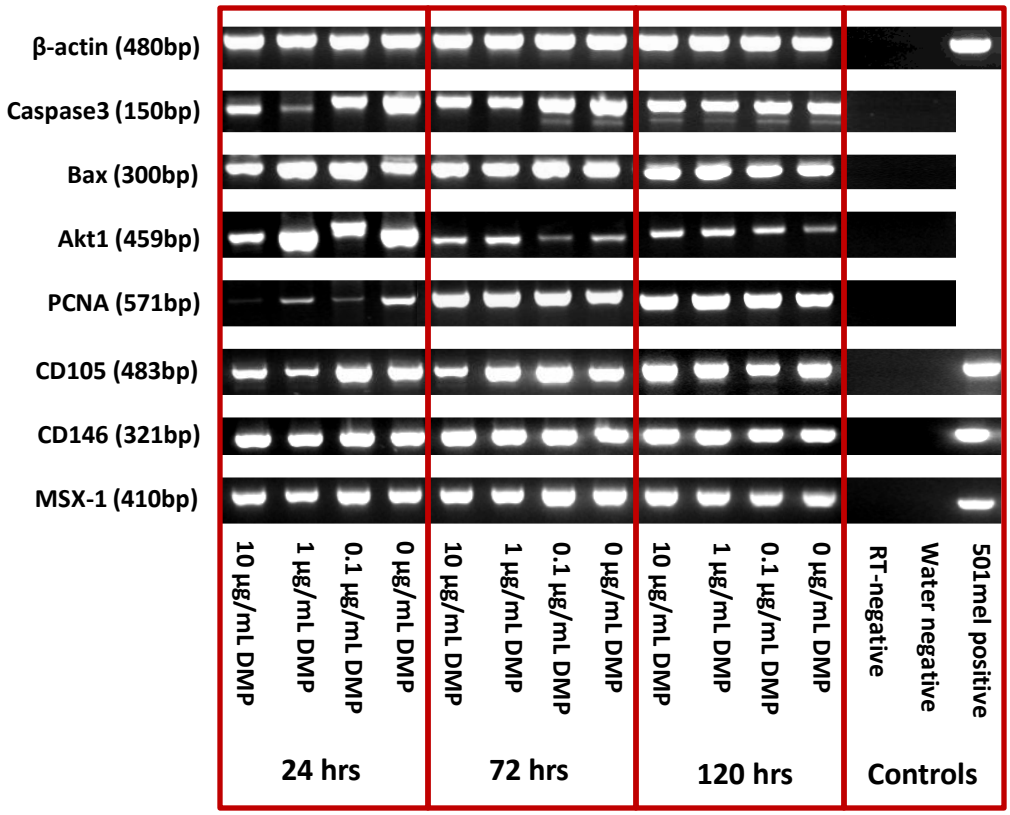



**Figure 4.6: DPSC expression of apoptotic markers Caspase and Bax, survival marker Akt1, proliferation marker PCNA and multipotency markers CD105, CD146 and MSX-1 after culturing in various DMP concentrations. RT-negative and water were used as experimental controls, and 501mel was used as multipotency positive control.**

**Table 4.2:** Continuous expression of markers for apoptosis, survival and mesenchymal cells by hFNA clone C4 cultured in DMP, expression maintained through to 120 hrs of culture. Housekeeping gene β-actin maintained by all cultures, and positive expression of 501 Mel control.

| Markers | | 24 hrs DMP (µg/mL) | | | | 72 hrs DMP (µg/mL) | | | | 120 hrs DMP (µg/mL) | | | | Controls | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 10 | 1 | 0.1 | 0 | 10 | 1 | 0.1 | 0 | 10 | 1 | 0.1 | 0 | RT–ve/ Water | 501 Mel |
| Housekeeping | β-actin | + | + | + | + | + | + | + | + | + | + | + | + | – | + |
| Apoptosis | Caspase 3 | + | + | + | + | + | + | + | + | + | + | + | + | – | n/a |
| | Bax | + | + | + | + | + | + | + | + | + | + | + | + | – | n/a |
| Cell Survival | Akt1 | + | + | + | + | + | + | + | + | + | + | + | + | – | n/a |
| Proliferation | PCNA | + | + | + | + | + | + | + | + | + | + | + | + | – | n/a |
| | | | | | | | | | | | | | | | |
| Mesenchymal | CD105 | + | + | + | + | + | + | + | + | + | + | + | + | – | + |
| | CD146 | + | + | + | + | + | + | + | + | + | + | + | + | – | + |
| | MSX-1 | + | + | + | + | + | + | + | + | + | + | + | + | – | + |

culture the varying concentrations of TGF-β1 were shown to mostly express all the apoptotic and survival markers. Mesenchymal marker expression was maintained throughout the culture period with the varying TGF-β1 concentrations (figure 4.7 and summarised in table 4.3).

Culture in DMP-Neg found the cells expressing most of the apoptotic, survival and mesenchymal markers similar to the control cultures even with concentrations of DMP-Neg up to 10 μg/mL (figure 4.8 and summarised in table 4.4). Overall, the results demonstrate culturing hFNA cells in DMP, TGF-β1 or DMP-Neg has no apparent effect on expression of apoptotic markers, with constant expression found virtually throughout culture whilst maintaining survival and multipotent mesenchymal markers. The 501 Mel positive control was only used as a stem cell marker control and not for apoptosis or cell survival, hence is illustrated as "n/a" in the tables 4.2, 4.3 and 4.4.

### 4.3.3. DMP influence on DPSC Migration

Immunofluorescently stained hFNA cells were found inside collagen gels of the Boyden chamber assay (figure 4.9a and b), where they would have migrated through the pores of membrane inserts (figure 4.9c and d) to reach the collagen gels. Confocal microscopy produced a stacked 3D image of cells (an example stacked image shown in figure 4.10a). Using the top-most visible cell as an arbitrary starting point the cells were shown to have migrated into the collagen gels when supplemented with TGF-β1 with 24 and 72 hrs of culturing. The cells demonstrated they were able to migrate up to 80 μm into the gels supplemented with 1 ng/mL TGF-β1 after 72 hrs of culture (figure 4.10b). A z-stack video of collagen gels

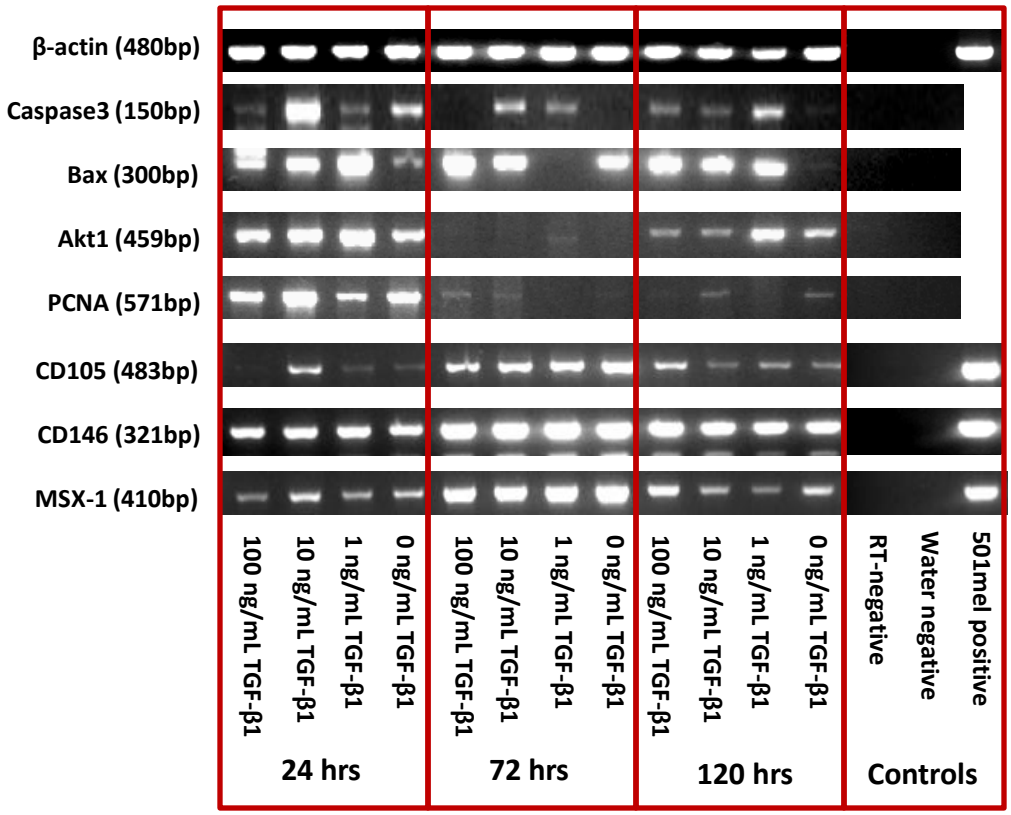



**Figure 4.7:** **DPSC expression of apoptotic markers Caspase and Bax, survival marker Akt1, proliferation marker PCNA and multipotency markers CD105, CD146 and MSX-1 after culturing in various TGF-β1 concentrations. RT-negative and water were used as experimental controls, and 501mel was used as multipotency positive control.**

**Table 4.3:** Expression of markers for apoptosis, survival and mesenchymal cells by hFNA clone C4 cultured in TGF-β1, expression appeared to be maintained through to 120 hrs of culture. Housekeeping gene β-actin maintained by all cultures, and positive expression of 501 Mel control.

| Markers | | 24 hrs TGF-β1 (ng/mL) | | | | 72 hrs TGF-β1 (ng/mL) | | | | 120 hrs TGF-β1 (ng/mL) | | | | Controls | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 100 | 10 | 1 | 0 | 100 | 10 | 1 | 0 | 100 | 10 | 1 | 0 | RT–ve/ Water | 501 Mel |
| Housekeeping | β-actin | + | + | + | + | + | + | + | + | + | + | + | + | – | + |
| Apoptosis | Caspase 3 | + | + | + | + | – | + | + | – | + | + | + | + | – | n/a |
| | Bax | + | + | + | + | + | + | – | + | + | + | + | + | – | n/a |
| Cell Survival | Akt1 | + | + | + | + | – | – | + | – | + | + | + | + | – | n/a |
| Proliferation | PCNA | + | + | + | + | + | + | – | + | + | + | – | + | – | n/a |
| | | | | | | | | | | | | | | | |
| Mesenchymal | CD105 | + | + | + | + | + | + | + | + | + | + | + | + | – | + |
| | CD146 | + | + | + | + | + | + | + | + | + | + | + | + | – | + |
| | MSX-1 | + | + | + | + | + | + | + | + | + | + | + | + | – | + |

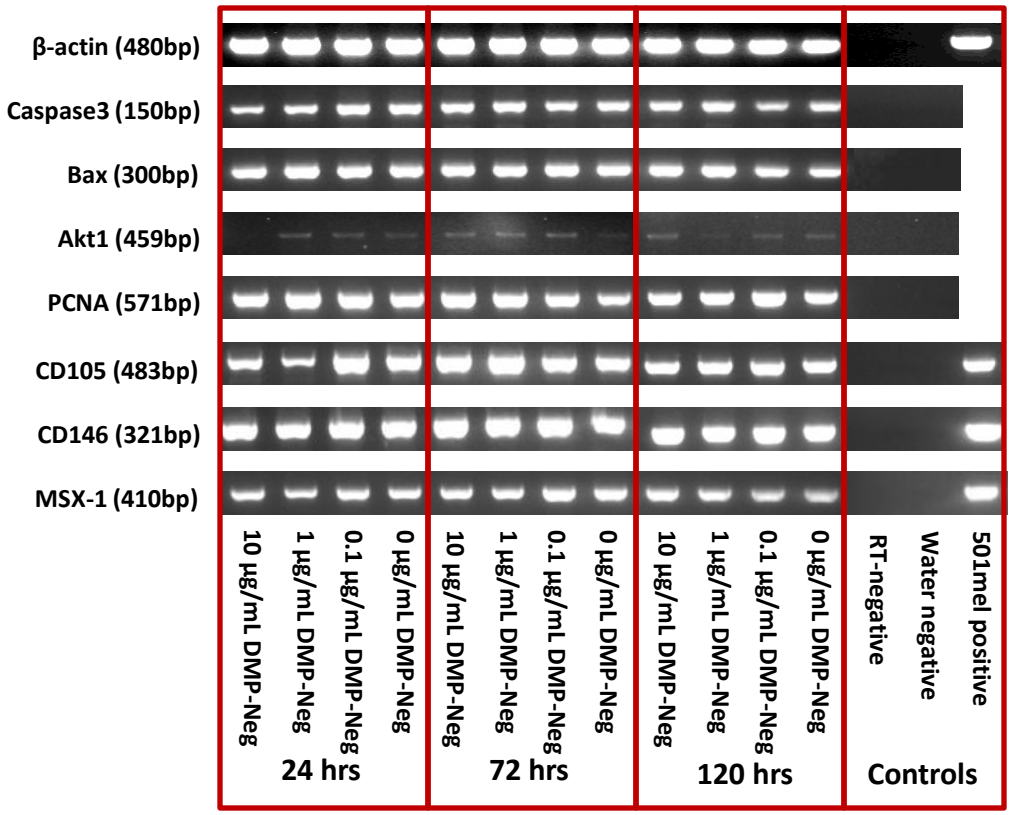



**Figure 4.8:** **DPSC expression of apoptotic markers Caspase and Bax, survival marker Akt1, proliferation marker PCNA and multipotency markers CD105, CD146 and MSX-1 after culturing in various DMP-Neg concentrations. RT-negative and water were used as experimental controls, and 501mel was used as multipotency positive control.**

Table 4.4: Expression of markers for apoptosis, survival and mesenchymal cells by hFNA clone C4 cultured in DMP-Neg, expression appeared to be maintained through to 120 hrs of culture. Housekeeping gene β-actin maintained by all cultures, and positive expression of 501 Mel control.

| Markers | | 24 hrs DMP-Neg (µg/mL) | | | | 72 hrs DMP-Neg (µg/mL) | | | | 120 hrs DMP-Neg (µg/mL) | | | | Controls | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 10 | 1 | 0.1 | 0 | 10 | 1 | 0.1 | 0 | 10 | 1 | 0.1 | 0 | RT–ve/ Water | 501 Mel |
| Housekeeping | β-actin | + | + | + | + | + | + | + | + | + | + | + | + | – | + |
| Apoptosis | Caspase 3 | + | + | + | + | + | + | + | + | + | + | + | + | – | n/a |
| | Bax | + | + | + | + | + | + | + | + | + | + | + | + | – | n/a |
| Cell Survival | Akt1 | – | + | + | + | + | + | + | + | + | + | + | + | – | n/a |
| Proliferation | PCNA | + | + | + | + | + | + | + | + | + | + | + | + | – | n/a |
| | | | | | | | | | | | | | | | |
| Mesenchymal | CD105 | + | + | + | + | + | + | + | + | + | + | + | + | – | + |
| | CD146 | + | + | + | + | + | + | + | + | + | + | + | + | – | + |
| | MSX-1 | + | + | + | + | + | + | + | + | + | + | + | + | – | + |

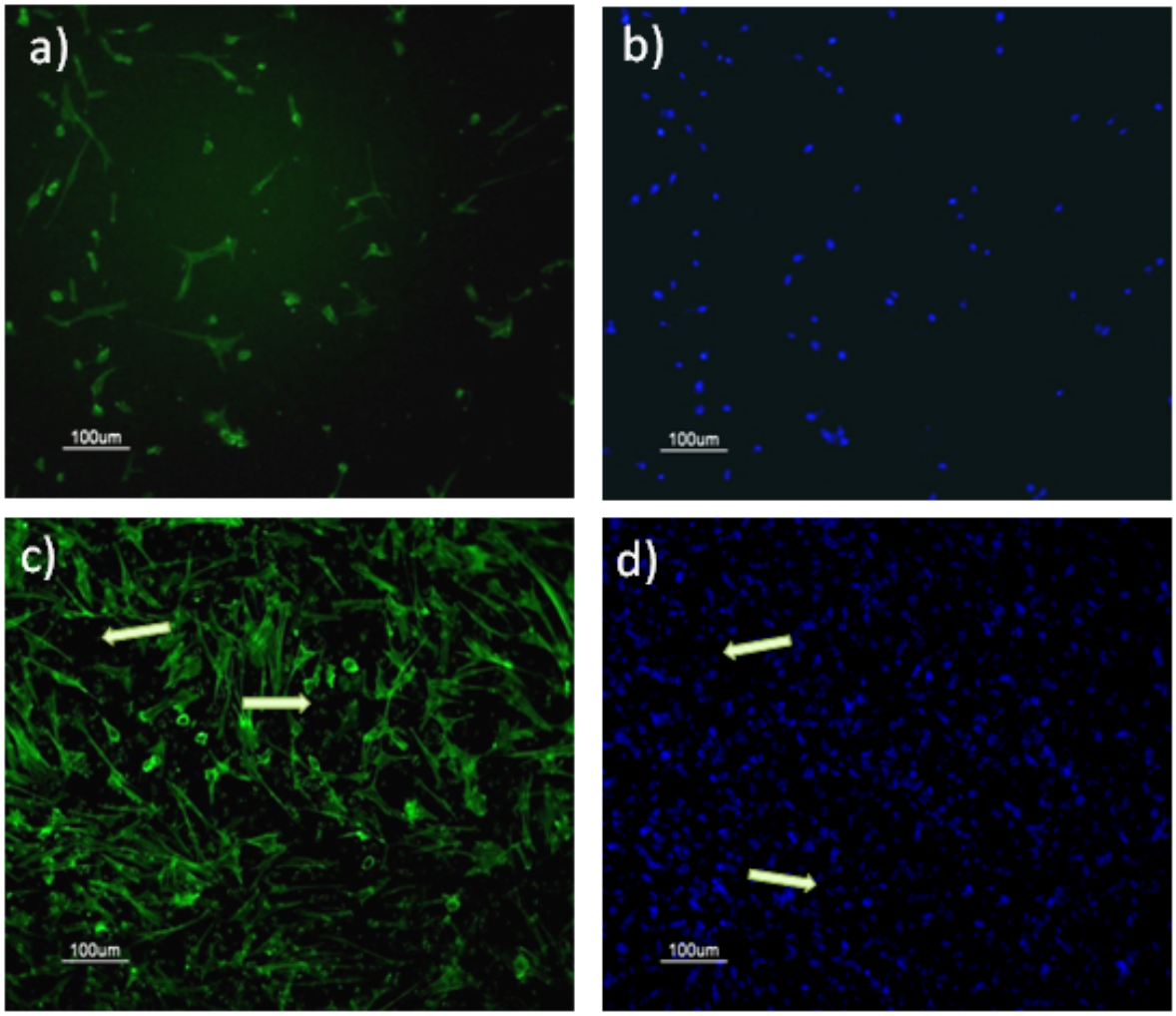



Figure 4.9 Immunofluorescence staining of hFNA cells within collagen gels for their actin cytoskeleton (a) and nuclei (b). Cells remaining on porous inserts were also stained for actin cytoskeleton (c) and nuclei (d), with the pores of the insert membrane clearly visible (arrowed). ×10 magnification, actin cytoskeleton stained green (phalloidin-FITC) and nuclei counterstained blue (bisBenzimide).

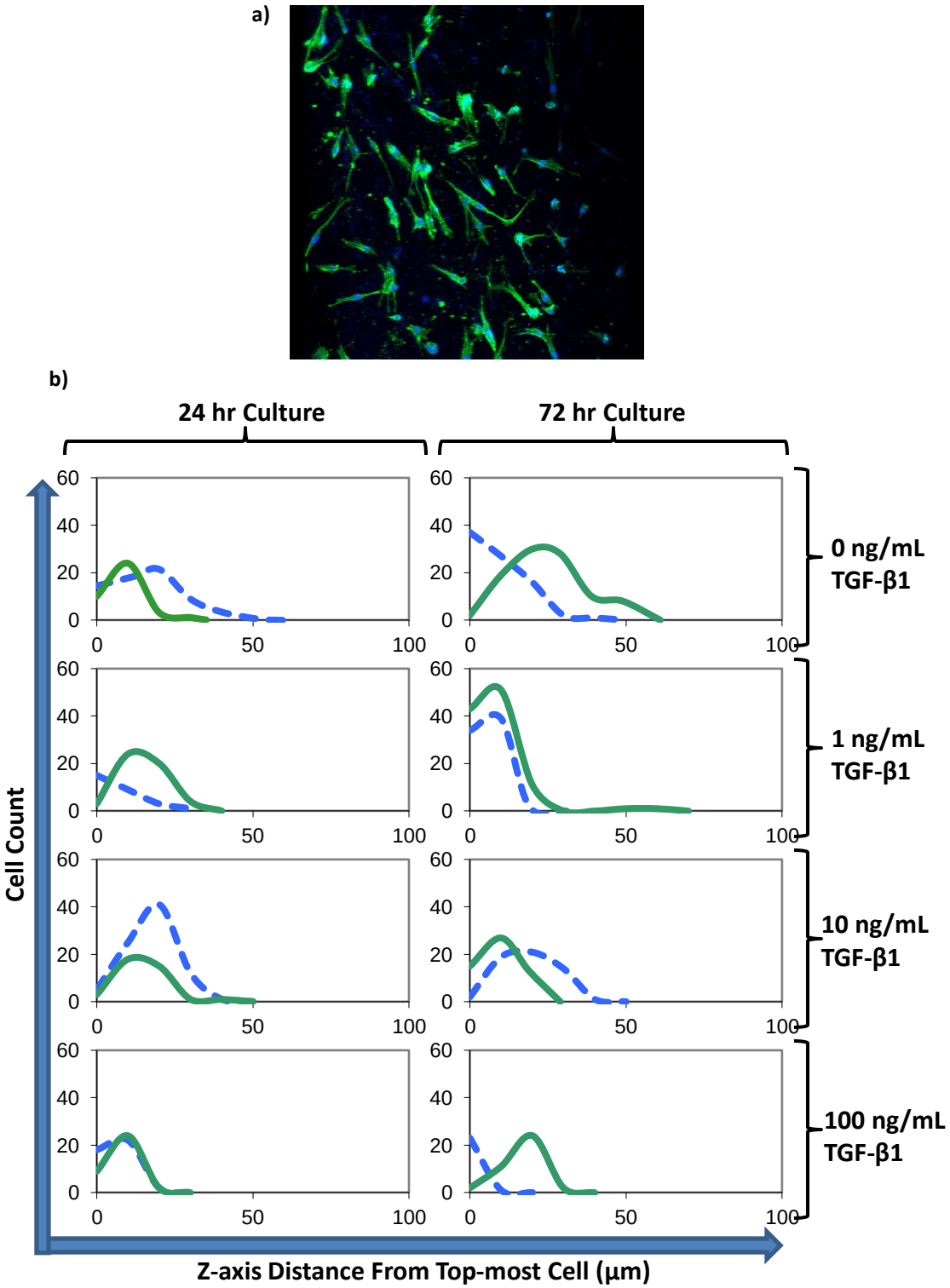
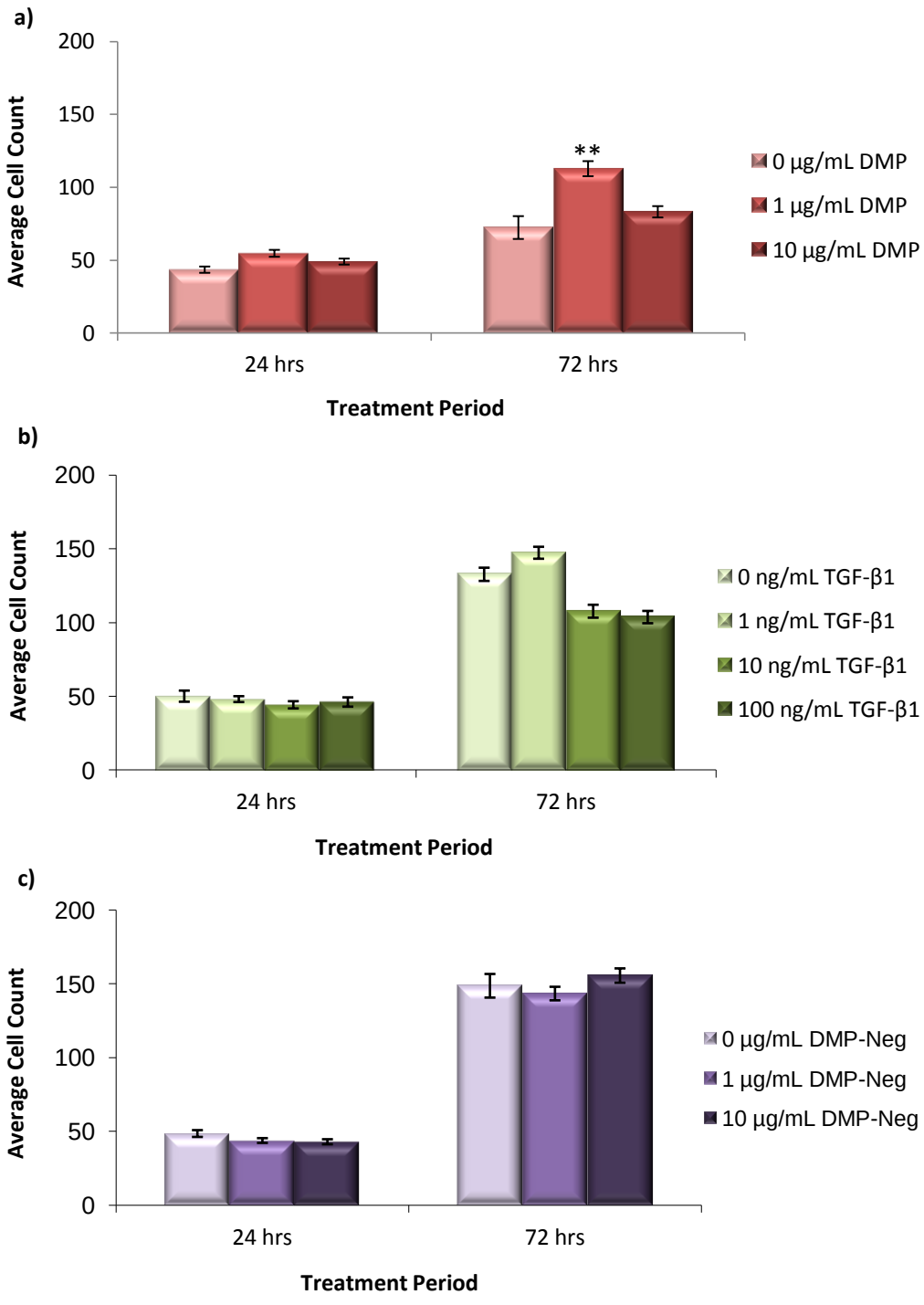



**Figure 4.10:** Confocal microscopy z-stack 3D image (×10 magnification) of hFNA cells cytoskeleton (green FITC) and nuclei (blue bisBenzimide) within a collagen gel (a). Cells were cultured for 24 or 72 hrs before taking confocal z-image sections, at 10 µm increments, to count cells as proof of migration into collagen gels with varying TGF-β1 concentrations (duplicate experiments represented by full and dashed lines) (b).

supplemented with 100 ng/mL TGF-β1 after 72 hrs demonstrated the presence of cells within the gels (supplementary CD video3 is a clip of the z-stack and video4 is the same stack of images but placed together to form a 3D image).

Average cell counts from five random fields of view provided a semi-quantification of hFNA cells present in the collagen gels, where more cells were identified in the gels after 72 hrs of culture compared with 24 hrs of culture as expected. A dose-dependant and time-dependant response was shown when supplementing DMP into the collagen gels. Culture with DMP for 24 hrs did not cause any changes in cell numbers when compared with the 0 μg/mL control cultures but only after 72 hrs were the differences significant. After 72 hrs culture, supplementing with 1 μg/mL DMP caused a highly significant increase in cell numbers (112.77 ±5.16 cells) compared with the 0 μg/mL controls (72.38 ±7.83 cells) while the 10 μg/mL DMP concentration did not produce a significant increase in cell numbers (83.20 ±3.88 cells) (figure 4.11a).

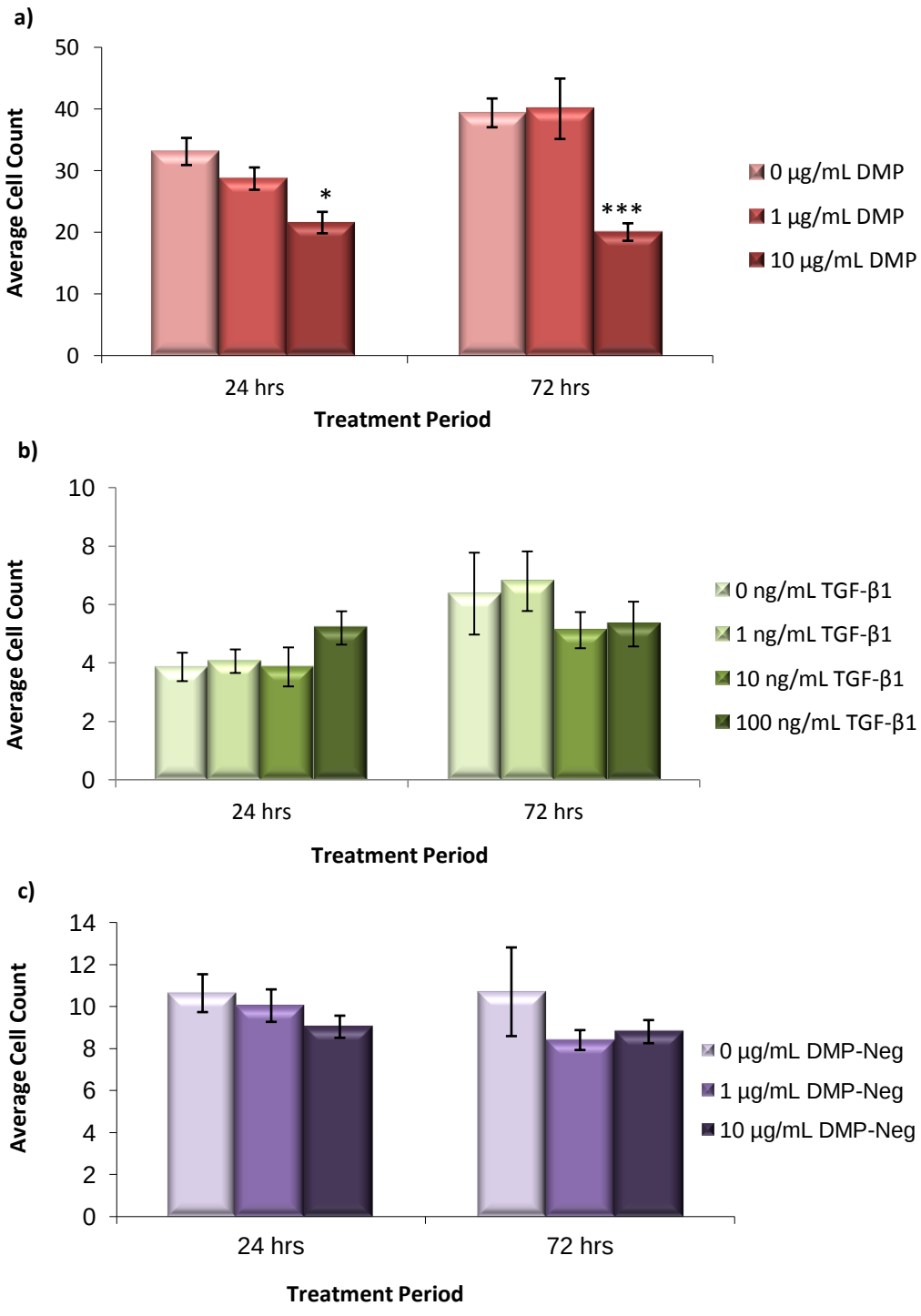
Collagen gels supplemented with various TGF-β1 concentrations demonstrated no significant changes in cell numbers compared with the 0 ng/mL controls even with up to 72 hrs of culture (figure 4.11b). The collagen gels supplemented with DMP-Neg also had no significant changes with cell numbers when compared with the 0 μg/mL control cultures even after 72 hrs of culture (figure 4.11c). These results indicated TGF-β1 and DMP-Neg has no apparent effect on the cell numbers that are present in the supplemented collagen gels, whereas the addition of lower concentration DMP demonstrated a significant increase of cell numbers in the gels with 72 hrs of culture.




**<u>Figure 4.11:</u> Comparison of cell counts of supplemented to unsupplemented Boyden gels, using hFNA cells and cultured for 24 and 72 hrs. Boyden gels supplemented with DMP (n = 9) (a), TGF-β1 (n = 6) (b), or DMP-Neg (n = 6) (c). Error bars represent SEM and ** = P <0.01.**

When the effect of DMP on cell migration was measured using a non-adherent hNA cell population, different results to the hFNA cells were observed. A dose-dependant response was shown by culturing in DMP supplemented gels. Culturing in 1 µg/mL DMP had no significant difference in cell numbers when compared to the 0 µg/mL control for both 24 and 72 hr cultures. Culture in 10 µg/mL supplemented gels there were significant decrease in cell numbers in the collagen gels. At 24 hrs there were a significant decrease in cell counts (21.06 ±1.74 cells) compared with the control (33.13 ±2.20 cells) and a very significant decrease at 72 hrs (20.07 ±1.42 cells) compared with the 72 hr control (39.40 ±2.33 cells) (figure 4.12a). Supplementing gels with TGF-β1 caused no significant changes in cell numbers at 24 hrs or 72 hrs of culture (figure 4.12b). Similarly, culture with DMP-Neg had no significant difference in cell numbers at 24 hrs or 72 hrs of culture (figure 4.12c). These results illustrated less cells were present in the collagen gels when using hNA cells compared with the hFNA population and supplementing with higher concentration of DMP reduced cell numbers although supplementing with TGF-β1 or DMP-Neg had no effect on cell numbers.

Preliminary DMP-Neg experiments used DMP treated with GuCl but without collagenase/dispase ("DMP-GuCl") (section 4.2.4). The hFNA cells supplemented with DMP-GuCl had no significant changes in cell numbers compared with the unsupplemented 0 µg/mL cultures after 24 hrs. Compared with the unsupplemented controls at 72 hrs there was a highly significant increase in cell numbers when supplementing with 1 µg/mL DMP-GuCl, and a significant decrease in cell numbers when with 10 µg/mL DMP-GuCl (figure 4.13). This demonstrated a dose-dependant and time-dependant response with supplementing with DMP-GuCl.




**<u>Figure 4.12:</u> Comparison of cell counts of supplemented to unsupplemented Boyden gels, using hNA cells and cultured for 24 and 72 hrs. Boyden gels supplemented with DMP (n = 3) (a), TGF-β1 (n = 6) (b), or DMP-Neg (n = 3) (c). Error bars represent SEM and * = P <0.05 and *** = P <0.001.**

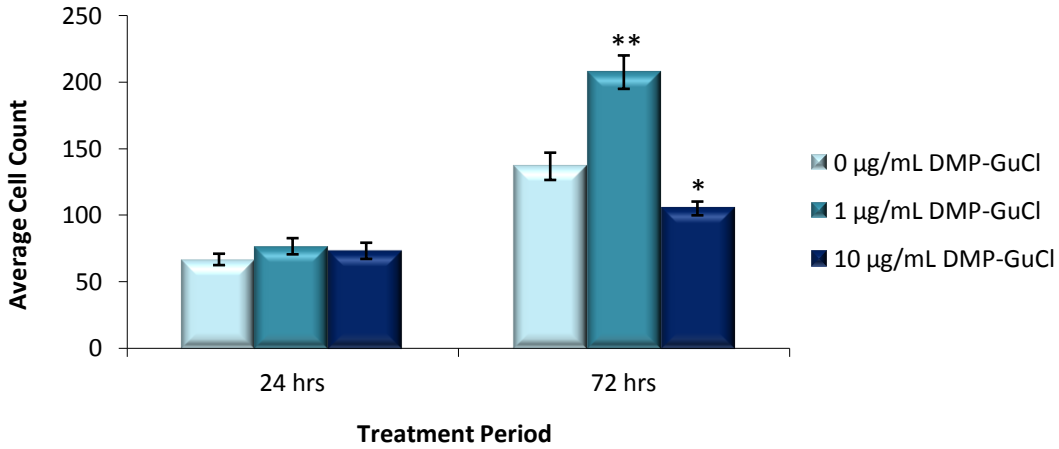



**Figure 4.13: Preliminary Boyden experiments supplemented with DMP treated with GuCl but without collagenase/dispase treatment. hFNA cells cultured for 24 and 72 hrs (n = 3) and cell counts compared with unsupplemented controls. Error bars represent SEM and * = P <0.05 and ** = P <0.01.**

## 4.4. Discussion

This study successfully demonstrated that DMP induces increased DPSC number expansion, maintains cell vitality, decreases apoptosis and has a dose-dependant and time-dependant effect on migration with *in vitro* culturing.

The DMP contains a cocktail of growth factors and cytokines with many influential roles on tooth development and repair (Bègue-Kirn et al. 1994; Finkelman et al. 1990). TGF-β1 is a well established growth factor present in dentine matrix with roles in tooth development, ECM secretion and the differentiation of DPSCs to odontoblast-like cells. Previous experiments using blocking antibodies to TGF-β1/2 abolished odontoblast differentiation (Begue-Kirn et al. 1992; Cassidy et al. 1997; Sloan and Smith 1999), making TGF-β1 ideal for use as a positive control. The DMP proteins/cytokines were assumed to be eliminated or denatured with GuCl treatment and collagenase/dispase digestion to produce a negative control (DMP-Neg). Ideally the DMP-Neg should have been verified by western blotting prior use in experiments to determine if protein fractions are present.

The collagenase/dispase digestion proved to be an essential step where preliminary studies with just GuCl treatment (DMP-GuCl) has shown significant changes in cell numbers with 72 hrs of culture, supplementing with 1 µg/mL had higher cell numbers present in collagen gels and 10 µg/mL had lower cell numbers when compared with the unsupplemented controls. The treatment with collagenase/dispase may aid denaturing of any proteins remaining and it is believed that the omission of the digestion step did not remove the collagen fibres that vastly make up the dentine matrix. Growth factors can be tightly bound or entrapped within the collagen fibres

(Butler and Ritchie 1995) and treatment with GuCl may simply reduce cytokine levels. The endogenous proteolytic enzymes (host collagenases and matrix metalloproteinases) or lactic acid secretion (*in vivo* pathogenic infiltration) naturally causes the release of entrapped growth factors from the collagen fibres (Cassidy et al. 1997; Dung et al. 1995).

Thus the preliminary work with only GuCl treatment may have shown a serendipity effect, where lowered concentrations of growth factors may have a greater stimulatory response. This coincides with previous findings where DMP had shown a pro-angiogenic response in a dose-dependant manner. Studies have demonstrated that DMP promotes angiogenesis when cultured with human endothelial cells, where reduction of 10 μg/mL to 0.1 μg/mL DMP increases efficacy for angiogenesis whilst 100 μg/mL or more DMP has an inhibitory effect (Zhang et al. 2011). This study suggested the cocktail of growth factors in DMP may have a synergistic effect and may lead to an amplification of cell responses when compared with using a single growth factor.

The MTS assay measures cell viability by utilising the cell's own (mitochondrial) dehydrogenases to break down the MTS assay substrate (Quent et al. 2010). The results indicated supplement with up to 10 μg/mL DMP did not affect DPSC vitality. As there were no significant differences between the DMP supplemented cultures and the unsupplemented control cultures, it was possible the DPSCs may have already been at a maximum metabolic rate. However culturing with TGF-β1, a known mitogen of immature cells (Pelton et al. 1991; Sloan and Smith 1999), caused an increase of MTS absorbance readings when compared with the unsupplemented

controls. This suggests the DPSCs were not at maximum activity and demonstrated the cells were able to respond to the growth factor, meaning they had possessed the appropriate receptors for cell signalling. The addition of DMP-Neg had no effect to the MTS absorbance readings when compared with the control cultures. This was expected as the GuCl and collagenase/dispase processing is assumed to have removed or denatured the existing cytokines of the DMP.

These results suggest that although DMP had not caused changes to DPSC cell vitality, DMP is known to have a role in odontoblast differentiation and cell migration (Demarco et al. 2010; Smith et al. 1994) where changes in cell behaviour, such as differentiation, may not be detectable with the MTS assay and should have been identified using odontogenic and osteogenic markers such as DSP, OCN and BSP. Maintenance of cell vitality with DMP supplementation illustrates the possible therapeutic role it may have when developing novel dentine repair strategies.

Crystal violet staining allowed for direct counting of viable cell numbers over time. DMP was shown to induce an increase of cell numbers when cultured for over 72 hrs, supporting previous studies (Finkelman et al. 1990) where DMP may cause progenitor cell number expansion during reparative dentinogenesis.

The addition of TGF-β1 had no apparent effects on cell numbers even with 72 hrs of culture. TGF-β1 was demonstrated with *in vitro* and *in vivo* studies to have a role in odontoblast differentiation (Begue-Kirn et al. 1992; Ruch et al. 1995; Smith et al. 1995). This suggests the cells supplemented with TGF-β1 may have undergone

differentiation instead of cell division, although this was not verified with osteogenic or odontogenic markers.

When the DMP was treated with GuCl and collagnease/dispase (DMP-Neg), it was assumed it had removed or denatured the cytokines/bioactive proteins. Culture with DMP-Neg demonstrated not to have an effect on cell numbers, where cell numbers were similar to unsupplemented control cultures. In summary these results demonstrated DMP has an ability to expand cell numbers *in vitro* compared with using a single growth factor TGF-β1, where DMP may be an asset for tissue engineering during cell culture expansion, or dentine repair therapy for increasing DPSCs during reparative dentinogenesis.

Supplementing DPSCs with DMP reduced apoptosis effects indicated by the reduction of caspase-3 enzyme activity. Culturing for up to 120 hrs (5 days) supplemented with DMP had no effect on the expression of apoptotic (Caspase 3/Bax) and survival (Akt1) gene markers, with consistent expression throughout culture. The expression of both markers may simply be an indication of normal cell turnover and if the markers expressed were actually being translated to functional components is undetermined, or perhaps there was a delayed response which could have been determined with longer culture. By examining caspase-3 activity, DPSCs cultured for up to 72 hrs with higher concentrations (2.5-10 µg/mL) of DMP had a reduced caspase enzyme activity. Although previous studies suggest the rapid influx of matrix proteins during dentine injury with severe carious lesions, may cause reduced regeneration *in vivo* possibly due to unregulated signalling which may induce apoptosis (Bjørndal 2001; Sloan and Smith 2007; Smith et al. 2005). There

was an apparent (but non-significant) increase in caspase activity with the lower concentration of DMP compared with the unsupplemented controls. This may be explained by the changing in protein conformation which may have an effect on its activity. An article has demonstrated that DPP (a DMP component) binding affinity to hydroxyapatite is affected by its concentration, where greater affinity occurs with high concentrations of DPP (Milan et al. 2006). So the addition of higher concentrations of DMP may have similar effects on caspase activity.

A study has also shown TGF-β1 (a component of dentine matrix) has a dose-dependant effect on early apoptosis with a mouse odontoblast-like cell line (He et al. 2005). However, this study was carried out *in vitro* where the DPSCs *in vivo* may be affected by pathogens associated with the carious lesions (Mutoh et al. 2009) and this study utilised an immature, multipotent cell line which may show a different response to the differentiated odontoblast-like cells used by He et al. (2005).

The dentine matrix contains pro-inflammatory cytokines including IL-2, IL-6 and IL-8, with anti-inflammatory cytokines which include IL-4, and IL-10 (Cooper et al. 2010). These cytokines have been shown to be secreted by odontoblast cells (Pääkkönen et al. 2007) and are assumed to be entrapped in the dentine matrix, although its presence in the DMP fractions were not verified prior this experiment. It was suggested that DMP may have a role in maintaining DPSC viability and regulates inflammation as the DPSCs migrate to the injury site and differentiate into odontoblast-like cells, where unregulated inflammatory responses may otherwise induce cell apoptosis and halt repair (Cooper et al. 2010; Rutherford and Gu 2000).

As mentioned earlier, TGF-β1 demonstrates a dose-dependant effect on apoptosis (He et al. 2005) but this study used an immature DPSC line and supplementing with TGF-β1 had not caused any changes to the caspase activity compared with unsupplemented control cultures. The DMP-Neg was assumed to have depleted/denatured cytokines where supplementing with DMP-Neg had not caused any changes to caspase activity and had a similar caspase result as the unsupplemented cultures. The maintenance of mesenchymal markers after 120 hrs with supplementation with DMP, TGF-β1 or DMP-Neg suggests the DPSCs had maintained the mesenchymal genotype. As DMP has been shown to induce differentiation of DPSCs into odontoblast-like cells *in vivo* (Smith et al. 1995; Smith et al. 1994), the occurrence of differentiation needs to be confirmed with osteogenic and odontogenic differentiation markers such as DSP, BSP and OCN. These results illustrate potential therapeutic role of DMP during dentine injury, as it demonstrates an ability to reduce cell apoptosis, and may be utilised to preserve cell viability at the dentine injury site.

Boyden chamber experiments demonstrated an increase of DPSC found in DMP supplemented collagen gels. The hFNA DPSCs used were around 20-100 μm in size (figures 2.1. and 4.7.) and were able to pass through the 8 μm pores of the Boyden chamber inserts without diffusing easily through the pores, which may give false readings. The cells were shown to migrate a distance of up to 50 μm into the collagen gels as observed by confocal microscopy (figure 4.10). As the cells were detected by fluorescence imaging, the top of the non-fluorescent collagen gels were not detectable, so the top-most cells were used as the arbitrary starting point to measure the amount of cell migration. The testing of the concept was investigated

using various concentrations of TGF-β1 for 24 and 72 hours of culture. Although TGF-β1 has a half-life of several hours, it has been demonstrated to have a knock-on effect such as increasing BMP levels (Margoni et al. 2011; Wegner et al. 2012). Culture for up to 72 hours is to determine if there is an increased effect with longer culture time.

The odontoblast-secreted dentine matrix is known to contain many matrix metalloproteinases (MMP) vital for matrix remodelling, which includes MMP-2, shown to be able to cleave Dmp-1 as part of dentinogenesis (Chaussain et al. 2009) and MMP-9, shown to be present during pulp inflammation (Tsai et al. 2005; Zehnder et al. 2011) where both play a role in tooth development (Goldberg et al. 2003). MMPs have also been shown to cause remodelling of collagen fibres (Amos et al. 2009; Stephens et al. 2001), which may alter the gel density and influence cell migration results.

The addition of DMP appeared to have a dose-dependant effect on DPSC migration. In comparison with the unsupplemented controls, after 24 hrs the 10 μg/mL DMP caused an increase in cell numbers where the 1 μg/mL DMP had an even higher cell number present. By 72 hrs the 10 μg/mL DMP supplemented cultures had slightly higher cell numbers than the unsupplemented control culture and the 1 μg/mL concentration had a highly significant increase in cell numbers compared with the unsupplemented control. This illustrates the lower concentration of DMP supplemented gels has more cells migrating into it compared with using a higher concentration of DMP.

The addition of TGF-β1 or DMP-Neg had no effect on cell migration compared with the unsupplemented control cultures. TGF-β1 is shown to have a dose-dependant effect on DPSCs, having a role in cell migration and also modulating odontoblast differentiation (Begue-Kirn et al. 1992; Dobie et al. 2002; Nakashima et al. 1994). The lack of cell numbers may be due to the multipotent DPSCs differentiating instead of migrating although the occurrence of differentiation with mineralisation markers such as osteocalcin, BSP and DSP were not determined.

When a non-adherent hNA cell population was used as comparison to the hFNA population, higher concentrations of DMP (10 μg/mL) had less cell numbers in the collagen gels when compared with the cell numbers shown by the hFNA cells. The 10 μg/mL DMP concentrations had significantly less cells migrating into the collagen gels at 24 hrs and very significant fewer cells at 72 hrs, whilst the lower concentration of 1 μg/mL had no significant changes when compared with the unsupplemented controls. This difference in behaviour to the hFNA cells suggests the hNA to be a different cell population since the dental pulp contains a myriad of progenitor cell populations (Fitzgerald et al. 1990; Waddington et al. 2009). The different cells have different responses to the same stimulus which may play a role in dentinogenesis.

The hNA cells were believed to be a more mature and differentiated population (section 2.3.6) and the addition of DMP may have caused these mature cells to secrete (mineralisation) matrix instead of migrating as like the more immature progenitors cell response. This would have been determined with RT-PCR for mineralisation markers such as osteocalcin, BSP and DSP with staining by alizarin

red S. The ability of DMP to increase cell numbers towards an injury site during dentine injury suggests DMP may have a therapeutic role in enhancing dentine repair.

This chapter demonstrated that DMP supplementation can alter human DPSC behaviour in cell vitality, function, proliferation and migration. The numerous cytokines and NCPs in the DMP may have synergistic roles in regulating the behaviour of DPSCs when compared with the use of a single growth factor such as TGF-β1 (Roberts et al. 1985; Sloan and Smith 1999; Smith et al. 1998). DMP was not shown to alter DPSC viability which suggests it had no detrimental effect to the DPSCs. Maintenance of viability imply the cells were maintained at their current state or the cells were directed down a different pathway such as differentiation into an odontogenic lineage as shown with previous studies (Smith et al. 1995; Smith et al. 1994). Caspase activity reduction with supra-physiological levels of DMP supports the viability results and demonstrates there may be a preservative role of DMP which may be vital during reparative dentinogenesis when DPSC migrate into an inflamed injury site (Rutherford and Gu 2000).

There was a delayed increase of DPSC proliferation with DMP supplementation with the most proliferation after 72 hrs of culture. In the same 72 hrs, there was an increase in cell numbers in DMP supplemented collagen gels. It is possible the cells may had simply proliferated and "spread" into the collagen gels, but as significantly more cells were present with the lower concentration of DMP-supplemented collagen gels, it suggests the DMP may have a chemotactic effect. This coincides with the natural reparative dentinogenesis process where the release of dentine matrix

proteins during dentine injury causes an increase of DPSCs which then migrate towards the injury site (Lacerda-Pinheiro et al. 2008; Smith and Lesot 2001; Smith et al. 1990), where a study suggests the DPP in the dentine matrix has an integral role in promoting DPSC migration (Yasuda et al. 2008).

Previous studies with implantation of DMP into exposed pulps have demonstrated a reparative dentinogenesis response suggesting a therapeutic potential for the development of novel dentine repair therapies aiding dentine bridge formation (Fitzgerald et al. 1990; Goldberg et al. 2009; Murray et al. 2002; Tziafas 2004). These results are performed *in vitro*, future work may consider utilising an *ex vivo* tooth slice model (Hasegawa 1989) to study DMP effects on DPSC behaviour *in situ*.

# Chapter 5:

# Development of Cell Localisation in an *Ex-Vivo* Tooth Slice Model

## 5.1. Introduction

It has been demonstrated that cell-matrix and cell-cell interactions play a significant role in odontoblast development, differentiation and dentine repair (Bègue-Kirn et al. 1994; Fuchs et al. 2004; Ruch et al. 1995; Scadden 2006; Smith et al. 1994). The dentine-pulp environment has a vast amount of signalling factors that affect DPSC behaviour. This includes the DPSC migration towards an injury site and differentiation into odontoblast-like cells as part of dentine repair (Fitzgerald et al. 1990; Goldberg et al. 2009; Tecles et al. 2005) (section 1.5).

The migratory and proliferative pattern of DPSCs in its natural three dimensional (3D) extracellular matrix (ECM) environment is not fully understood. The majority of dentine repair studies were based on *in vitro* monolayer cultures (Couble et al. 2000; Gronthos et al. 2000; Nakashima et al. 1994; Waddington et al. 2009; Yu et al. 2010), *in vitro* 3D cultures (Iohara et al. 2004) or *in vivo* cultures that required sacrificing and fixing of samples for histology (Batouli et al. 2003; Fitzgerald et al. 1990). *In vitro* culturing demonstrated to be unable to fully replicate the natural microenvironment and ECM composition, where the lack of regulatory signals is suggested to cause changes in cell behaviour (Patel et al. 2009; Yu et al. 2010). 3D cell pellet culture or with the use of scaffolds appeared to enhance cell function such as odontoblast differentiation from DPSCs (Demarco et al. 2010; Iohara et al. 2004). Previous tissue engineering experiments that have utilised synthetic scaffolds (e.g. poly-L-lactic acid) and natural scaffolds (e.g. gelatin, bone and collagen) illustrated

improved cell adhesion and proliferation (Liu and Ma 2004, 2009), although these scaffolds lack natural ECM components so they do not replicate the complex *in vivo* microenvironment needed for the development of regenerative therapy. *In vivo* culturing makes it difficult to follow cell behaviour in real-time, requiring separate samples for different time points before sacrificing animals and fixing samples.

An established *ex vivo* organotypic culture model using mature tooth slices (Hasegawa 1989; Sloan et al. 1998) provides a complex 3D environment of the dentine-pulp complex for the study of cell behaviour during dentine repair. Previous experiments included cytotoxity testing of dental materials, effects of mechanical forces and addition of morphogens for tissue engineering experiments (Demarco et al. 2010; Dhopatkar et al. 2005; Murray et al. 2000; Sloan and Smith 1999). Work within our department had shown that the 3D organotypic culture model can be used to maintain cell morphology, tissue architecture and tissue viability for up to 21 days (Sloan et al. 1998; Smith et al. 2010). Maintenance of the distinct dental architecture and its compartments is a valuable asset for dental manipulation and investigating the dentine repair processes.

This chapter aims to develop a reproducible method for the localisation of fluorescently stained DPSCs transplanted into the organotypic tooth slice model. The fluorescently stained progenitors will be microinjected into the pulp or pulp-dentine interface of the tooth slice and will be assessed if they remain viable and locatable. The ability to follow DPSC behaviour in their complex environment will provide further insight into dentine repair and to study DPSC behaviour by manipulation of the tooth slice model.

## 5.2. Materials and Methods

### 5.2.1. Cell Source

The isolated DPSC clonal populations, hFNA3 clone C4 (27 PDs) and rFNA2 clone B2 (29 PDs) were shown to express mesenchymal and embryonic-like gene markers during early culture (figure 2.12 - 2.19), where the hFNA population also demonstrated multipotent differentiation characteristics (section 3.3).

### 5.2.2. Culture Medium

Culture medium was composed of alpha-modification Minimum Essential Medium (αMEM) pre-supplemented with ribonucleosides and deoxyribonucleosides (Gibco, Invitrogen, UK), and was further supplemented with hi-FCS, L-glutamine, L-ascorbate 2-phosphate and antibiotics/antimycotics as previously described (section 2.2.2).

### 5.2.3. Production of Tooth Slices from Rodent Incisors

Male Wistar rats (*Rattus norvegicus*) at 28 days old were supplied by Harlan Laboratories (UK), as previously described (section 2.2.1). Following sterilisation in 70% IMS the lower mandibles were separated using a scalpel and the soft mucosa surrounding mandibles were detached before the mandibles were dislocated and removed from the mouth. The remaining soft tissue surrounding the mandibles was cleared away using a scalpel. A sterile diamond-edged rotary bone saw was used to separate and discard the condyle, ramus and molars before the mandible sections were cut at approximately 2 mm thickness (figure 5.1). The bone saw cutting procedure was kept cool using sterile PBS and the tooth slices were held in antibiotic-treated αMEM before being used in the following procedures.

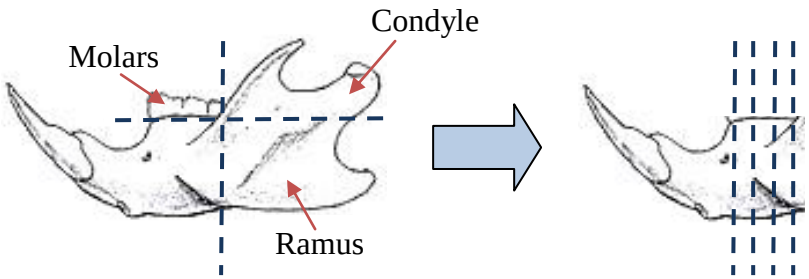



**Figure 5.1: Rodent mandible preparation and 2 mm sectioning using rotary bone saw. Dashes represent cuts made by bone saw.**

### 5.2.4. Viability Assessment of *Ex-Vivo* Cultured Mandible Slices

Mandible slices in triplicate were cultured in a 48-well tissue culture plate (Greiner Bio-One, UK) submerged in 2 mL culture medium at 37 ℃ and 5% $CO_2$. Following 2 and 7 days of culture the slices were treated with a live/dead stain composed of 100 µg/mL acridine orange and 100 µg/mL ethidium bromide (both Sigma-Aldrich, UK) in plain αMEM (Gibco, UK) for 2 s before it was washed briefly in PBS. Slices were then immediately transferred onto a microscope slide and viewed using a UV microscope (section 2.2.8) at 373 nm/456 nm (same as FITC wavelength) to view live (green) or dead (red) cells. The viewed slices were then treated with the live/dead stain for 10 mins until the ethidium bromide devitalised any cells that were present. These were then viewed under the same microscope conditions as "dead" control slices for comparison to any live cells present in the experimental samples.

### 5.2.5. Incorporation of PKH26 Tracker Dye into Monolayer Culture of DPSCs

A serum-free suspension of $1 \times 10^5$ DPSCs cells was pippetted into 4 sterile 1.5 mL eppendorfs (Eppendorf, UK), these were centrifuged at 400 ×g to produce cell pellets and the supernatant was discarded. Two cell pellets were resuspended in 25 µL of

Diluent C (iso-osmotic aqueous solution) and 25 µL of dye mix (249 µL Diluent C and 1 µL $1\times10^{-3}$ M PKH26 dye mixed and used immediately) as a $2\times10^{-6}$ M final dye concentration (PKH26 red fluorescent cell linker kit from Sigma-Aldrich, UK). The remaining 2 cell pellets were used as control samples where the PKH26 tracker dye was replaced with Diluent C. The 4 resuspended pellets were incubated at room temperature for 7 mins with regular agitation before washing 3× 300 µL serum-containing culture medium by centrifugation at 400 ×g. The cells were resuspended in 100 µL culture medium to give $1\times10^{3}$ cells/µL, where the two 50 µL volumes ($5\times10^{3}$ cells each) were each transferred into a separate well of a 6 well plate (Greiner Bio-One, UK). 2 mL of culture medium was added to each well and cultured for up to 7 days.

After 2 and 7 days of culture, the 2 wells of labelled cells and the 2 wells of the unlabelled control cells were viewed at 541 nm/572 nm (TRITC wavelength) on a argon laser microscope (DeltaVision, Applied Precision Inc, UK, with an Olympus IX71 inverted microscope and automated stage, UK) to determine the presence of tracker dye with imaging software softWoRx 4.0 (Applied Precision Inc, UK). After viewing the cells the medium was aspirated and 1 mL of lysis buffer was added by pipette (RLT Buffer, Qiagen, UK) before viewing again on the same fluorescence microscope to check for tracker dye presence after cell lysis.

### 5.2.6. Microinjection of PKH26 Stained DPSCs into Mandible Slices

The microsyringe used was a NanoFil syringe with a 35 Gauge bevelled needle (NanoFil, World Precision Instruments, UK) which was UV sterilised prior use. A total of 18 mandible slices were prepared for a 2 day culture and another 18 slices for

a 7 day culture. Using a single cell suspension of DPSCs in serum-free medium, $1x10^5$ cells were centrifuged into pellets and labelled with the PKH26 dye as previously described (section 5.2.5). The labelled cells were resuspended in 100 µL culture medium and 1 µL ($1\times10^3$ cells/µL) was used to inject into the mandible slices. Using a dissection microscope, for both 2 and 7 day time points, 6 mandible slices had labelled cells injected into the pulp region, 6 slices had labelled cells injected at the dentine-pulp interface region (figure 5.2) and the remaining 6 slices were sham injected with only culture medium at the pulp. These injected slices were cultured submerged in 2 mL culture medium in 48-well tissue culture plates (Greiner Bio-One, UK) at 37 ℃ and 5% $CO_2$.

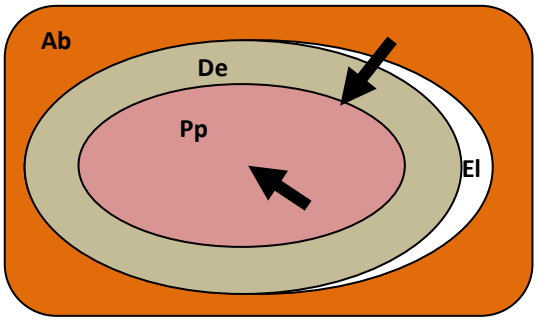



**Figure 5.2: Diagram of the microinjections sites (arrowed) of a typical tooth slice. Pp = pulp, De = dentine, El = enamel and Ab = alveolar bone.**

Following a culture period of 2 or 7 days, the labelled cells within the slices were imaged at 541 nm/572 nm (TRITC wavelength) with a UV microscope (section 2.2.8). Slices were then immediately fixed in 10 mL of 10% (w/v) neutral buffered formalin at room temperature with gentle agitation for at least 24 hrs. The fixed slices were then demineralised in 10 mL 10% (w/v) formic acid at room temperature with gentle agitation for 72 hrs. Slices were then placed into embedding cassettes for

automatic processing, paraffin embedding, sectioned to 5 μm thickness before being mounted on poly-L-lysine coated glass slides (SuperFrost) and stored until use. Sections were then rehydrated as previously described (section 3.2.5.2) for immunolabelling for the Stro-1 antigen.

### 5.2.7. Immunolabelling DPSCs for Stro-1

The rehydrated mandible sections (section 5.2.6) were circled using immiscible ink (Super PAP Pen, Daido Sangyo, Japan) before they were blocked with 1% fraction V BSA-TBS blocking buffer for 30 mins. Sections were then treated with either a mouse raised anti-Stro-1 IgM monoclonal antibody (Hybridoma Bank, USA) at 1:25 in blocking buffer, an IgM isotype control (GeneTex, USA) or with blocking buffer (primary antibody exclusion) for 1 hr at room temperature. These were then washed 5 times with TBS before the addition of goat raised anti-mouse IgM-FITC (Santa Cruz, Germany) at 1:500 dilutions and bisBenzimide at 5 μg/mL in same blocking buffer for 45 mins in darkness at 4°C. The sections were then washed for 5 mins with 70% IMS and 5 mins with xylene before the addition of Fluorsave (Calbiochem, Merck Chemicals, Germany) for mounting with a glass cover-slip. The samples were kept in darkness and at 4°C overnight and viewed the next day. Microscopy was carried out using an ultra violet (UV) microscope as previously described (section 2.2.8) at 373 nm/456 nm (FITC) and 490 nm/520 nm (bisBenzimide).

## 5.3. Results

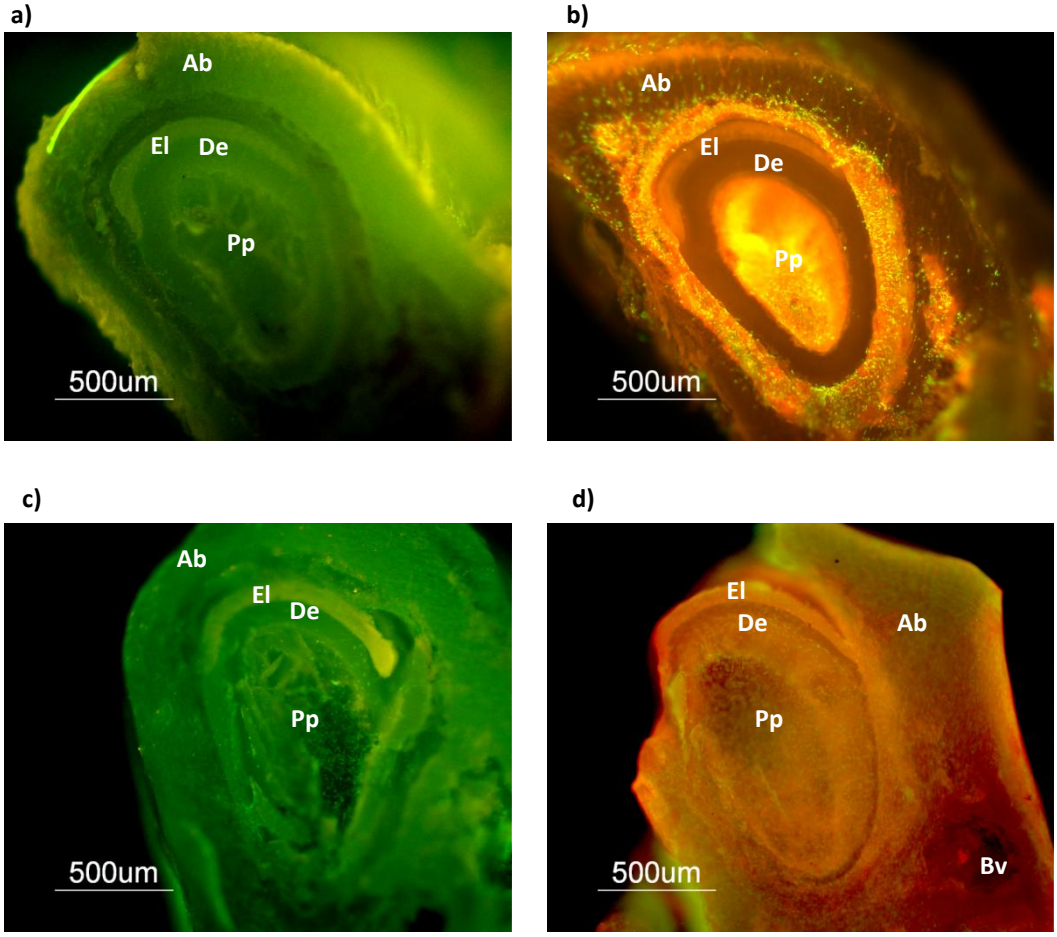
### 5.3.1. *Ex Vivo* Cultured Mandible Slice Viability

The mandible slices that were cultured submerged in medium remained viable for up to 7 days of culture. After 2 days of culture the treatment with live/dead staining illustrated the mandible slices had strong green fluorescence indicative of cellular viability (figure 5.3a). It had also maintained the tissue architecture with the pulp, dentine and enamel organs remaining intact. The day 2 control slices that had undergone cellular necrosis lost their vitality with an orange/red fluorescence that was indicative of cell death (figure 5.3b). Mandible slice viability and architecture were also maintained after 7 days of culture (figure 5.3c) when compared with its control slices (figure 5.3d).

### 5.3.2. Evaluation of PKH26 Stained DPSCs in Monolayer Culture

Both rodent and human DPSCs were stained with PKH26 tracker dye without apparent adverse effects. rFNA cells indicated that the PKH26 tracker dye has bound to the cell surface which remained present after 7 days of culture (figure 5.4a and b). The hFNA cells were also successfully stained and maintained fluorescence for up to 7 days of culture (figure 5.4c and d). Following the addition of tracker dye to rFNA and hFNA cells, both cell populations maintained a fibroblast-like morphology, a similar morphology to the unstained control cells which suggested that the use of tracker dye did not affect cell morphology.

As a test to observe the effects of cell necrosis on the PKH26 staining, the lysis of labelled cells ultimately eliminated fluorescence caused by the tracker dye. It appeared that when the tracker dye was bound at a high concentration to a cell




**<u>Figure 5.3:</u>** ***Ex-Vivo*** **cultured rodent mandible sections vitality live/dead staining after 2 days (a) and its dead control (b), and up to 7 days (c) and its dead control (d). Green fluorescence represent the viable cells, whilst red/orange fluorescence represent non-viable cells. Images taken at ×4 magnification, where Pp = pulp, De = dentine, El = enamel, Ab = alveolar bone, and Bv = blood vessel.**

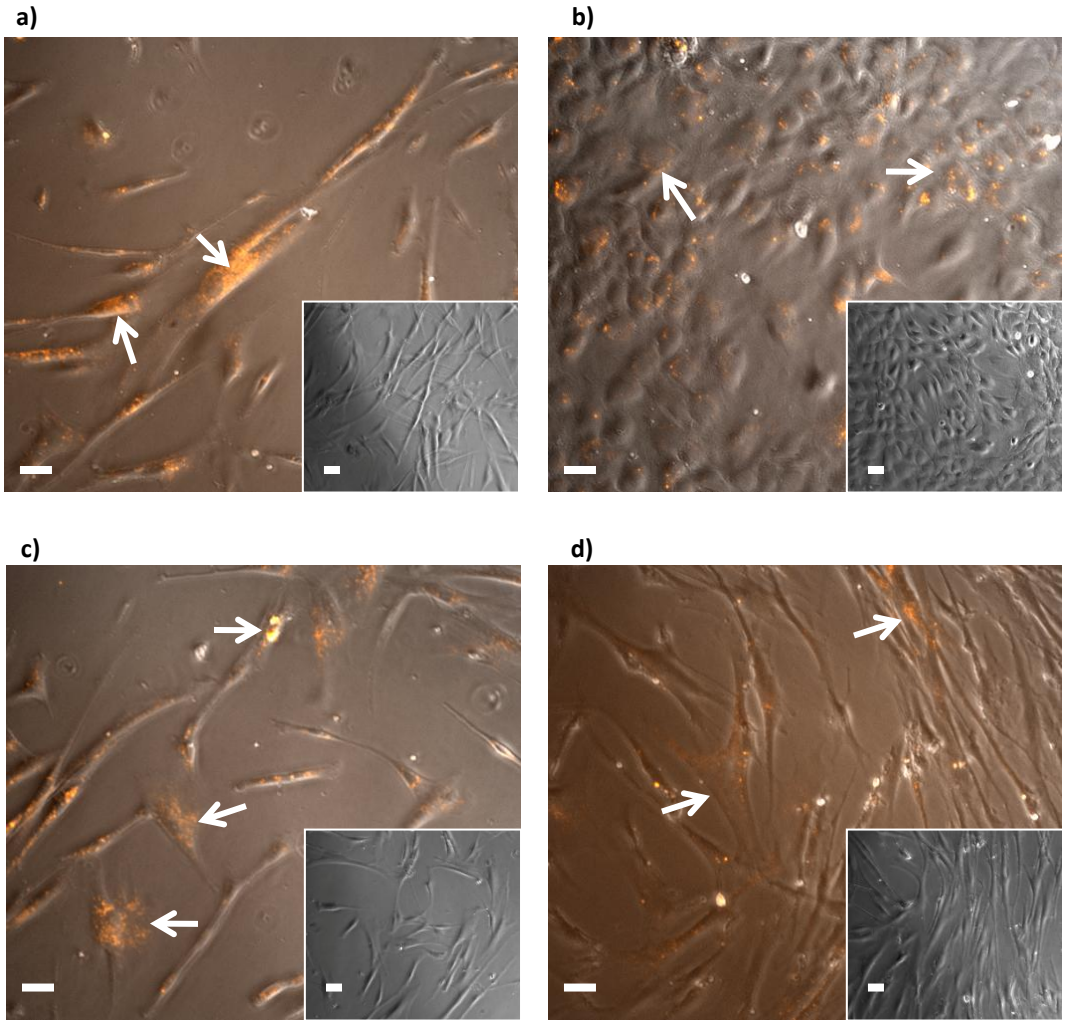



**Figure 5.4:** **Preliminary PKH26 stained DPSCs cultured as monolayer, using rodent rFNA cells for 2 days (a) and 7 days (b), and human hFNA cells for 2 days (c) and 7 days (d). ×10 magnification images of fluorescent dye bound to cell membranes (arrowed) and the insets represent unstained cell controls. Bars represent 100 µm.**

surface the fluorescence would become visible (figure 5.5a). The unstained control cultures were non-fluorescent before and after lysis (figure 5.5b).

### 5.3.3. Microinjection of Rodent DPSCs into Mandible Slices

The PKH26 stained rFNA were injected into the dental pulp (figure 5.6a) and the pulp-dentine interface (figure 5.6b). Fluorescent cells that were found in clusters at the injected locations all maintained fluorescence with 7 days of culture and higher magnification images revealed the cells had a fibroblastic morphology. The control mandible slices indicated no presence of fluorescent cells (figure 5.6c) although it brought to attention the autofluorescence of the mineralised tissues (dentine and enamel). Autofluorescence is commonly witnessed with fluorescent imaging, suggested due to NCP fluorophores within the mineralised structures (Foreman 1988; Lin et al. 2010).

The immunohistochemistry of the mandible sections revealed dense Stro-$1^{+}$ clusters at injected sites, this further confirmed and strengthen the findings of the PKH26 stained cells localisation, which were present at 2 and 7 days of culture (figure 5.7a and b). However, the sham injected control slices (figure 5.7c) revealed an expression of Stro-$1^{+}$ cells throughout the sections, not just at the injected sites, albeit less densely labelled (figure 5.7c). Immunohistochemistry controls with primary antibody exclusion (figure 5.7d) or substituted with an isotype control (figure 5.7e) were Stro-$1^{-}$. Some of the tissues from the sections were washed away during the immunohistochemistry procedure therefore were absent from some of the images.

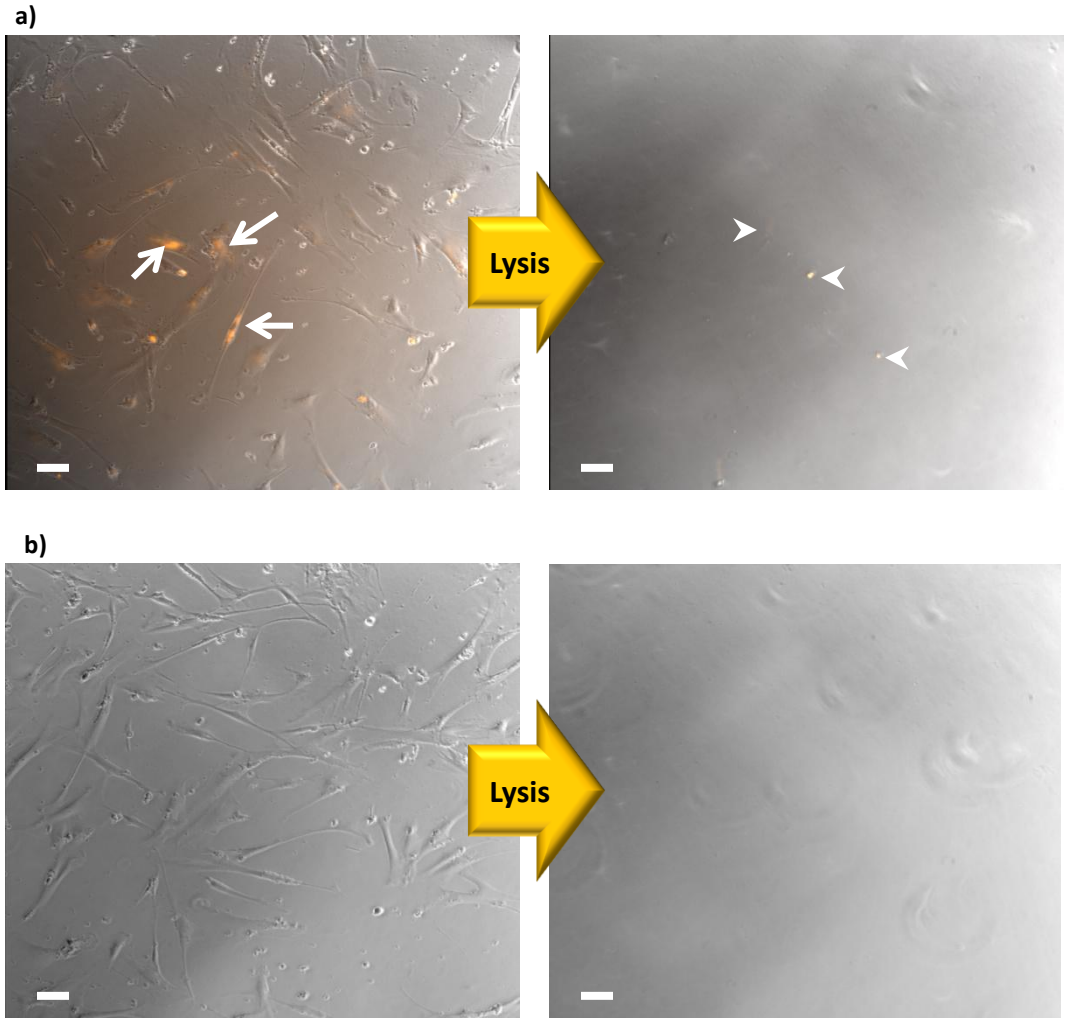



**Figure 5.5: Lysis of PKH26 stained hFNA cells results in major loss of fluorescence (a) and the unlabelled control cells remained non-fluorescent (b). ×10 magnification images of fluorescent cells illustrated by arrows and residual unlysed cells illustrated by arrowheads. Bars represent 100 µm.**

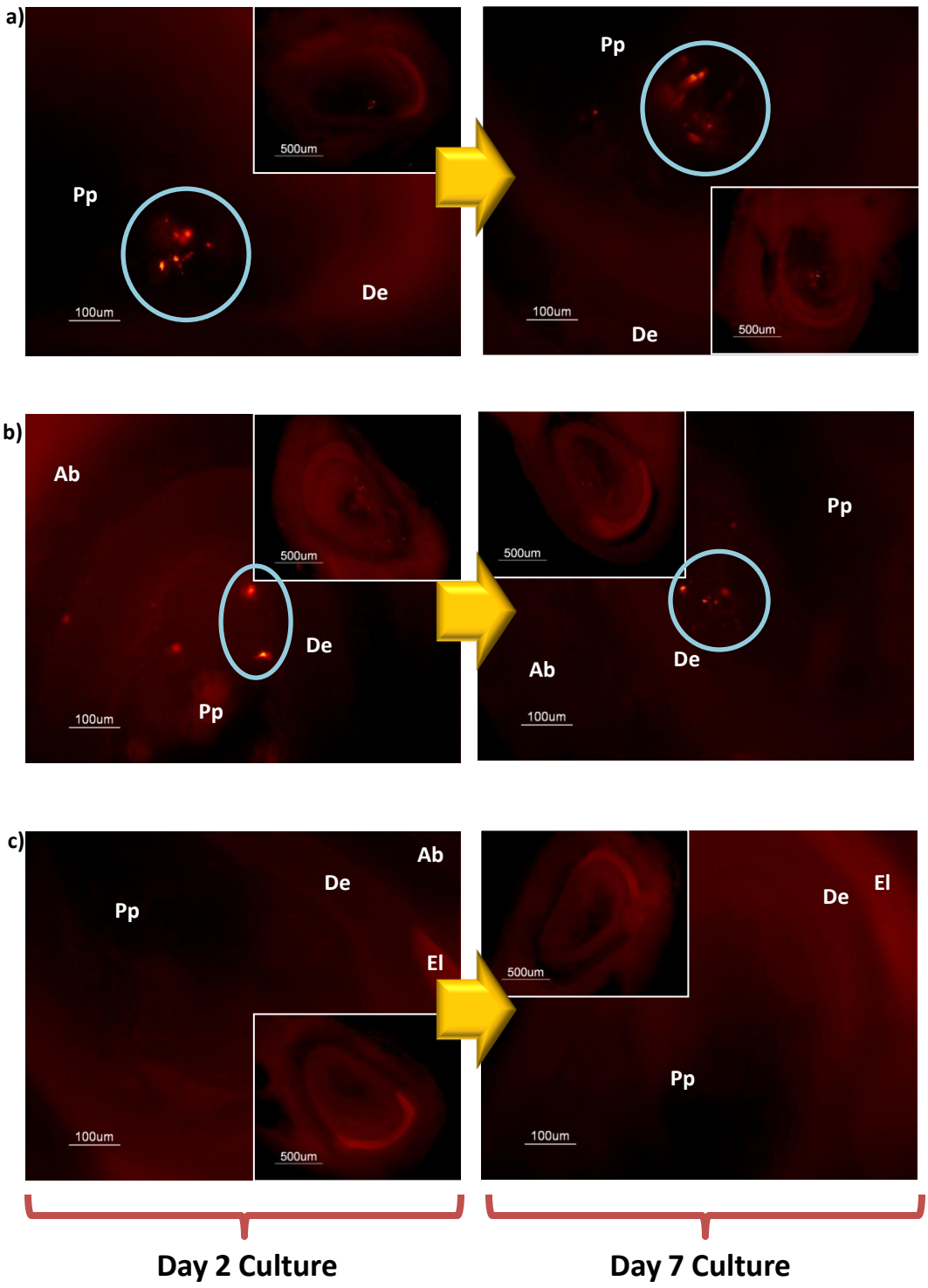



**Figure 5.6:** Day 2 and day 7 cultures of PKH26 labelled rFNA cells (circled) microinjected into the rodent tooth pulp (a), dentine-pulp interface (b) or sham injected into the pulp (c). Illustrating a dispersion of cells with day 7 culture. Images taken at ×10 magnification, and insets are its ×4 magnification. Pp = pulp, De = dentine, El = enamel and Ab = alveolar bone.

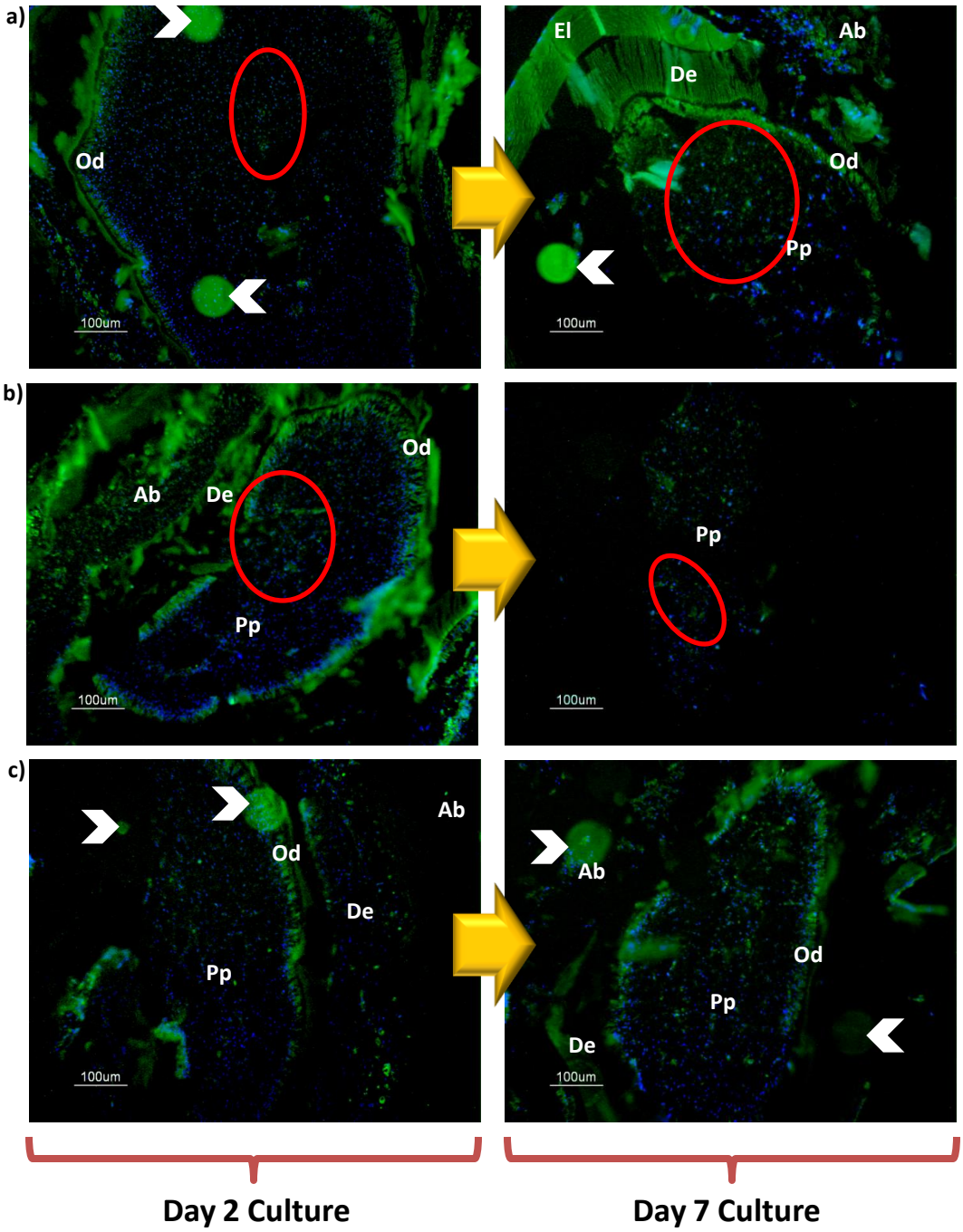
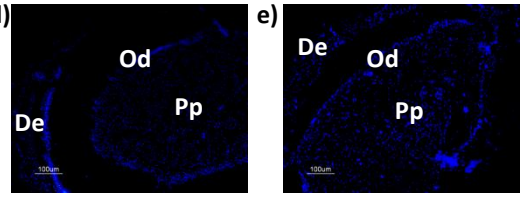



**Figure 5.7:** Day 2 and day 7 rFNA Stro-1$^+$ cells in rodent mandible slices form high density clusters (circled). Microinjected into pulp (a), dentine-pulp interface (b) or sham control (c). Controls primary exclusion (d) and isotype control (e). ×10 magnification images, green FITC = Stro-1$^+$, blue bisBenzimide = nuclei, Pp = pulp, Od = odontoblasts, De = dentine, El = enamel and Ab = alveolar bone. Air bubbles (arrowheads) are artefacts from tissue preparation.



### 5.3.4. Microinjection of Human DPSCs into Mandible Slices

The injection of PKH26 stained hFNA cells into the dental pulp (figure 5.8a) and pulp-dentine interface (figure 5.8b) showed a dense clustering of fluorescent cells 2 days post-injection. By day 7 of culture the fluorescent cells were more dispersed with cells that appeared to have spread out and became less densely concentrated. This may be an indication of cell proliferation and diffusion of the fluorescent dye as the cell divided, or possibly the effects of dye degradation. The control slices had no fluorescent cells present which were as expected (figure 5.8c).

The immunohisochemistry of mandible sections revealed dense areas of cells that were Stro-$1^{+}$ at the pulp (figure 5.9a) and dentine-pulp interface (figure 5.9b), but with no dense clusters of Stro-$1^{+}$ cells with the sham injected slices (figure 5.9c). This correlated with the microinjected sites of PKH26 stained progenitor cells, although there was a similar positive expression throughout the mandible sections similar to the rFNA injected slice sections (figure 5.7). Experimental control sections with primary antibody exclusion (figure 5.9d) or used an isotype control (figure 5.9e) remained Stro-$1^{-}$.

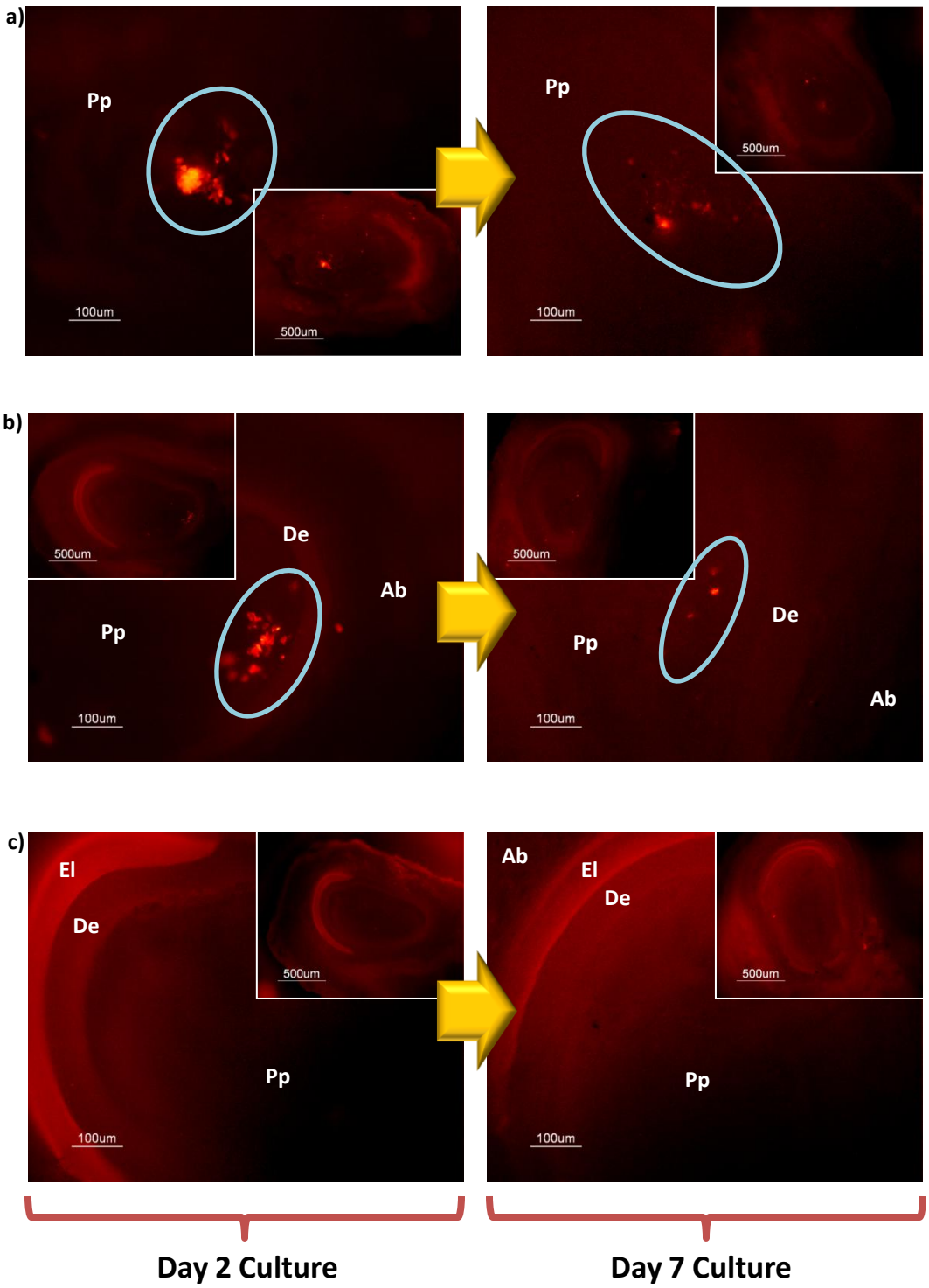



**Figure 5.8:** Day 2 and day 7 cultures of PKH26 labelled hFNA cells (circled) microinjected into the rodent tooth pulp (a), dentine-pulp interface (b) or sham injected into the pulp (c). Illustrating a dispersion of cells with day 7 culture. Images taken at ×10 magnification, and insets are its ×4 magnification. Pp = pulp, De = dentine, El = enamel and Ab = alveolar bone.

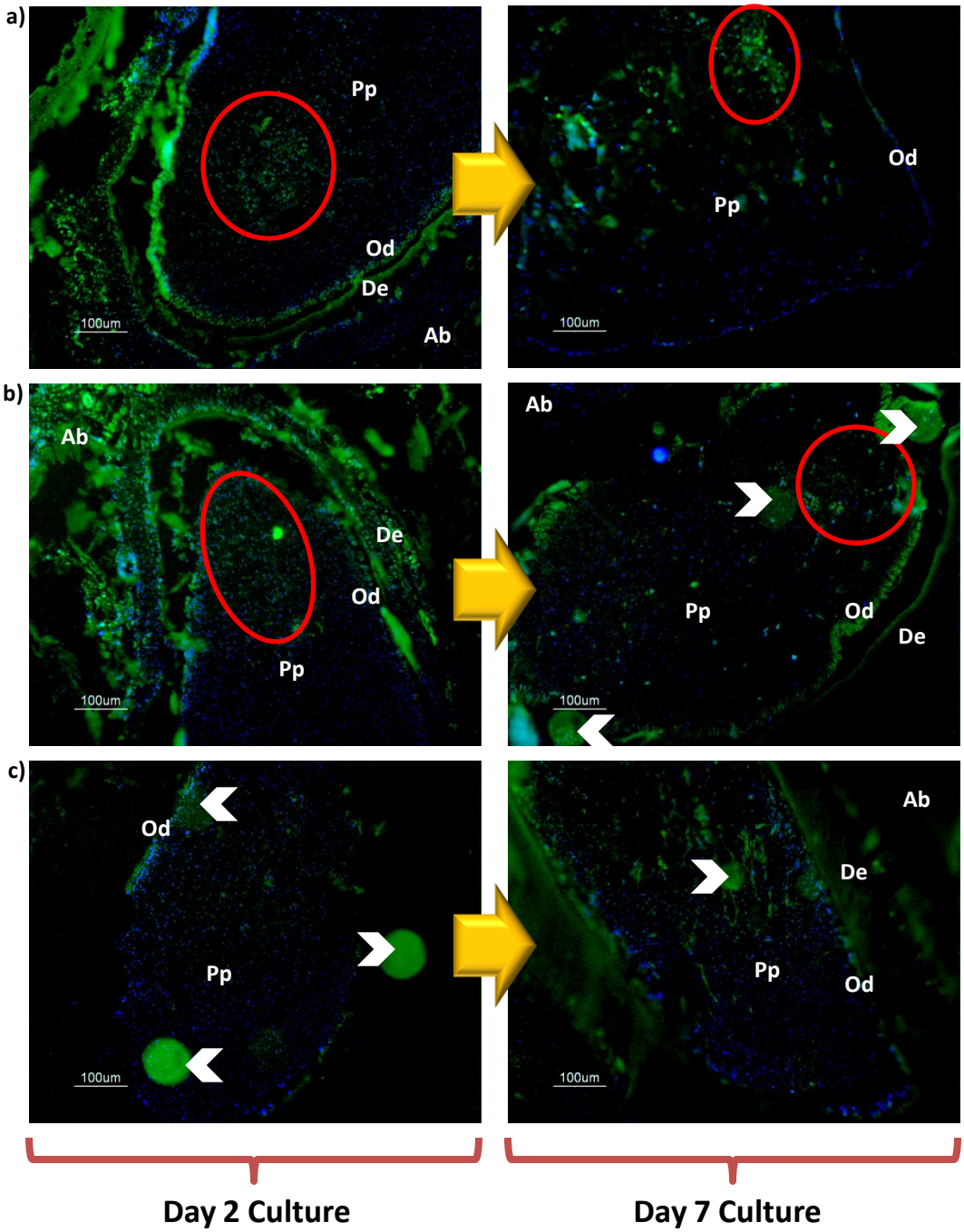
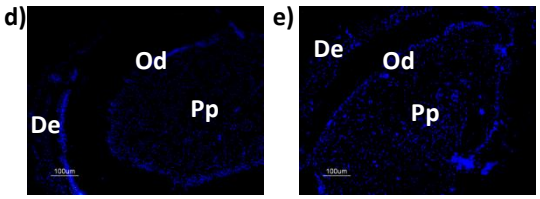



**Figure 5.9:** Day 2 and day 7 hFNA Stro-1$^+$ cells in rodent mandible slices form high density clusters (circled). Microinjected into pulp (a), dentine-pulp interface (b) or sham control (c). Controls primary exclusion (d) and isotype control (e). ×10 magnification images, green FITC = Stro-1$^+$, blue bisBenzimide = nuclei, Pp = pulp, Od = odontoblasts, De = dentine, El = enamel and Ab = alveolar bone. Air bubbles (arrowheads) are artefacts from tissue preparation.



## 5.4. Discussion

This study has successfully utilised an *ex vivo* model to enable the study of DPSC behaviour in its tooth environment. The mandible slice model and the microinjection of stained DPSCs into the slices allowed for cell localisation and possibly for its migration or proliferation.

The cut mandible slices remained viable and were able to maintain distinct tooth architecture (pulp, dentine layer, enamel, bone) even after 7 days of culture when submerged in culture medium, which validated its capability for use as a tooth model (Hasegawa 1989). Maintaining the viability of mandible slices allows it to be used as a model for dental manipulation (Smith et al. 2010) and the maintenance of the tooth layers are important for replicating the niche.

Studies have previously shown the loss of the dentine matrix component can cause columnar odontoblasts to take on a more fibroblast morphology and affect reparative dentinogenesis (Batouli et al. 2003; Demarco et al. 2010; Smith et al. 1994). Whilst the pulp has been suggested to have a role in nutrient transport with the diffusion of nutrition and gases, proven to be important in a 3D environment especially for use in an organotypic culture model (Hasegawa 1989). Future experiments may require longer culturing time (e.g. to allow for progenitor cell differentiation) and a trowel-type system may be more ideal. A trowel-type culture positions the mandible sections at a liquid-gas interface to increase oxygen tension and nutrient circulation, this helps to promote longer periods of culture to help maintain tissue vitality and cell phenotype (Hasegawa 1989; Sloan et al. 1998; Smith et al. 2010).

The fluorescent PKH26 tracker dye successfully bound to the DPSC membrane and the fluorescence was only visible in intact cells. Successful PKH26 staining was shown with the monolayer culture test where the stained cells appeared healthy with a fibroblast-like morphology. The lysis of the fluorescent cells caused a loss of detectable fluorescence. As PKH26 binds to the cell membrane it was debated whether stained cells undergoing necrosis within the tooth environment would remain fluorescent, which would have caused false positive readings. After cell lysis the natural turnover and circulation of the tissue would remove any labelled cellular debris (Pinzon et al. 1966). This suggested that the visible fluorescence of the PKH26 tracker dye was from viable cells only and can be used for locating stained cells, suggesting a possible use in assisting the study of cell behaviour within the tooth slice model. Staining the DPSCs with PKH26 dye would enable the localisation of the cells after they were injected into the mandible slice which would be beneficial for studying cell behaviour such as migration or proliferation in the tooth.

The microinjection of the PKH26 stained rodent rFNA or human hFNA cells into specific areas of the tooth slice, such as the pulp or dentine-pulp interface, was shown to be successful by the clustering of fluorescent cells which lasted up to 7 days of culture. Immunolabelling of the Stro-$1^{+}$ cells illustrated a dense collection of positive cells at the injection sites compared with the less densely labelled background fluorescence. Taken together it demonstrated successful microinjection of cells into the desired areas of the tooth, enabling the study of DPSC behaviour in response to the tooth environment.

Culturing of the injected mandible slices for 7 days showed the stained cells had dispersed in the tooth slice environment. This may be due to cell proliferation where the membrane-bound fluorescent dye was "diluted" with cell division or by the migration of the stained cells across the tissue (or perhaps even both). This would have been revealed if time lapse microscopy had been to be used, which would have tracked the stained cells for a time period to determine cell proliferation and/or cell migration. The dispersion of fluorescence was greatest by the hFNA cells, where it was understood that the differences in cell populations between the rFNA and hFNA (sections 2.3.6 and 2.3.7) may have contributed to the differing fluorescence dispersion characteristics, but importantly these results demonstrated the injected cells were locatable, viable and were able to function within the tooth slice model.

Stro-1 has been successfully used as a marker to identify multipotent cells (Yang et al. 2007a; Yang et al. 2007b; Yu et al. 2010; Zhang et al. 2005), and was subsequently used to identify microinjected DPSCs in the tooth slices. The results demonstrated positive expression of Stro-1 throughout the tooth tissue (particularly in the pulp), suggesting an anomalous result which may be due to inadequate serum blocking or excess antibodies not being washed away. Nonetheless, dense areas of fluorescence were distinguishable and correlated with the injection sites.

The method of sawing mandible sections may have caused the resident progenitors from the pulp to migrate towards the exposed cut surfaces (towards an injury site) as a reparative dentinogenesis response (Fitzgerald et al. 1990; Tecles et al. 2005). Expression of Stro-1 throughout the sections may have been due to the mouse-raised monoclonal antibody cross-reacting with the rat tooth, highlighting the presence of

multipotent progenitors that are Stro-$1^+$ residing within the pulp (Shi and Gronthos 2003; Yang et al. 2007b; Yu et al. 2010).

The injection of human cells into a rodent tooth slice (xenogenic transplant) may cause immunological rejection which may play a role in altering cell behaviour. The inflammatory response was not examined, for example the immunodetection of proinflammatory cytokines interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α in the tooth slices (Colombo et al. 2011). This would be an important issue if a rodent tooth slice is to be used with human cells as a model for studying DPSCs.

However, if the isolated DPSCs were MSC-like, it has been suggested MSCs have immunoprivileged properties which would not cause an inflammatory response (Huang et al. 2010; Rastegar et al. 2010). Alternatively it is possible to create a tooth slice model using a human tooth, although this is less convenient to use as a model due to ethical reasons, variation in patient age/health/genes (Ma et al. 2009) and to collect enough teeth from the same patient for comparative experiments.

The tooth slice model and the ability to locate fluorescently stained DPSCs allowed the study of cell behaviour to be done *in situ*, a methodology that has vast potential for the study of DPSCs during dentine injury. The tooth slice environment can also be manipulated to assist in srudying the behaviour of DPSCs when growth factors (e.g. TGF-β1) or DMP are added.

This chapter was able to successfully show that DPSCs can be labelled with PKH26 tracker dye for localising after microinjection into a viable tooth slice model. The

cells were shown to remain viable and may be able to migrate or proliferate within the tissue, although this was not determined in this chapter. These results verified that the tooth slice model could be utilised with human or rat DPSCs for up to 7 days and PKH26 was acceptable for cell localisation experiments in the tooth slice, although any inflammatory responses that may occur will need to be clarified. Time-lapse microscopy and perhaps the use of alternative tracking agents could allow development of better cell tracking procedures. This would allow investigation of DPSC function during reparative dentinogenesis and during manipulation of the tooth environment, such as the addition of cytokines (e.g. TGF-β1 and DMP).

# Chapter 6:

# General Discussion

## 6.1. Discussion

The investigation of DPSC commitment and behaviour provides the potential to enhance dentine repair strategies and for its use in tissue engineering experiments. This thesis set the task to isolate and characterise distinct populations of DPSCs by fibronectin preferential selection and with MACS to isolate P75 neural crest cells. The morphological features, growth kinetics and expression of stem cell genetic markers were compared to distinguish both isolates as distinct populations. An immature population was selected for differentiation into adipogenic, chondrogenic and osteogenic lineages to assess its differential potential and to define a multipotent DPSC population for investigation into dentine repair. As DMPs are naturally released during dentine injury, the effects of DMP in modulating DPSC viability, proliferation and migration were examined. Positive DPSC responses to DMP stimulation may suggest its potential role in the development of dentine repair therapy. Additionally, the DPSCs were utilised to develop a method to monitor cell behaviour when injected into a tooth slice. This provides a better representation of the local niche environment compared with *in vitro* studies of DPSCs during dentine repair. The understanding of DPSC behaviour during dentine injury will provide a better foundation for developing treatment to enhance the clinical outcomes of dentine regenerative therapy.

The presence of multiple and distinct populations of progenitors within the dental pulp is well acknowledged (Huang et al. 2009; Iohara et al. 2009; Waddington et al.

2009). This thesis reveals the distinct populations are capable of forming colonies and are morphologically dissimilar. The isolation of fibronectin adherent (FNA) and neural crest $P75^{+}$ progenitors from the dental pulp yielded fibroblast-like cells. However, the $P75^{+}$ cells were distinctly smaller when compared with the more oblong-like NA and $P75^{-}$ populations (figure 2.1).

Both FNA and $P75^{+}$ populations demonstrated a greater aptitude to form colonies when compared to the NA and $P75^{-}$ populations (sections 2.3.2 to 2.3.4). This suggests the FNA and $P75^{+}$ populations were not only distinct but were more immature compared with the NA and $P75^{-}$ populations, where the greater proficiency to form colonies has been previously demonstrated by immature progenitors (Huang et al. 2009).

The sustained PD growth kinetics further supports that immature populations were isolated, where clonal populations of DPSCs provides a more consistent growth kinetic. There were differences in PDs between the mixed heterogeneous FNA and NA cultures where the hNA and rFNA had far superior sustained PDs compared with hFNA and rNA respectively (figures 2.5 and 2.9). The clonal isolates derived from the same experiment, the FNA compared to the NA or the $P75^{+}$ compared to $P75^{-}$ populations had similar PD patterns (figures 2.6 - 2.8 and figures 2.10 - 2.11). These results demonstrated there were variable levels of cell maturity, or perhaps cell commitment, within the mixed heterogeneous populations.

Isolating clones derived from the earliest forming colonies had most likely selected for the most immature cells in both positively selected (FNA and $P75^{+}$) and

negatively selected (NA and $P75^{-}$) populations, therefore both would display similar growth kinetics. This supports the idea that the adult dental pulp can also harbour a more immature population of cells, which may include the more proliferative SCAP population (Lei et al. 2011; Sonoyama et al. 2008).

To confirm cell commitment and immaturity, the gene expression of the isolated populations were observed. The whole dental pulp had expression of several multipotent mesenchymal markers and pluripotent embryonic markers (table 2.3 and 2.4) suggesting the presence of immature and possibly undifferentiated cells. This supports the previously mentioned growth kinetic results where the proliferative progenitors are most likely to be immature progenitors (Huang et al. 2009). There were differences in gene marker expression between the FNA and $P75^{+}$ populations (table 2.3), highlighting the differences between the cell commitment and maturity within the pulpal populations.

The gene markers maintained during early *in vitro* culturing (around 25 PDs) appeared to be absent after around 100 PDs (50 passages) where the loss of markers may occur earlier, as previous work demonstrated stem cell marker loss as early as 25 PDs (Kerkis et al. 2006). This loss of marker expression may be due to the cells undergoing spontaneous differentiation and perhaps senescence as suggested by previous literature (Patel et al. 2009). The lack of extracellular signalling factors *in vitro* that are naturally present within the dental niche may affect regulation of cell division and differentiation kinetics (as discussed through sections 1.7 - 1.9) which may have a role in maintaining the stem cell markers.

With the isolation of an immature and undifferentiated DPSC population, cell culture expansion may be required to increase cell numbers for further experimentation. Future long-term culture of the undifferentiated DPSCs may consider utilising extracellular factors to determine if it would maintain it in an immature state. An example extracellular factor includes the use of Wnt and LIF proteins which were shown to suppress MSC differentiation (section 1.6.2.2). Maintaining an undifferentiated phenotype would be valuable for bulk expansion of immature undifferentiated DPSCs for tissue engineering use.

An alternative procedure to maintain the DPSCs in an immature state is to consider utilising iPS technology to modify the DPSCs into a pluripotent cells. The embryonic marker Nanog and one of the major pluripotency factors Oct4 was shown to be present in the isolated DPSCs (table 2.3). Gene expression of Sox2, Klf4 and c-Myc needs to be determined then the DPSCs may be exploited by iPS (section 1.6.1). Progenitors from the dental tissue had already demonstrated the ability to form pluripotent progenitors with a higher efficacy than fibroblast cells, making them valuable for iPS study and tissue engineering (Meissner et al. 2007; Takahashi et al. 2007; Yan et al. 2010).

For the human dental pulp, the clonal hFNA population expressed the most multipotency and pluripotency gene markers in comparison with the heterogeneous hFNA and the $hP75^{+}$ populations. This suggested the hFNA clone was the most immature and uncommitted population for use with differentiation experiments, which would determine its potential and prospective uses in dental repair and tissue engineering experiments.

The isolated immature hFNA clonal population was differentiated into the mesenchymal adipogenic, chondrogenic and osteogenic lineages (section 3.3). Much of the literature has utilised a heterogeneous DPSC population which differentiated into one or few mesenchymal tissues and even cells with neurogenic characteristics (Gronthos et al. 2000; Kadar et al. 2009; Yu et al. 2010).

The multipotent differential capacity of the hFNA clone demonstrated the plasticity of the isolated DPSCs, making it a valuable adult stem cell population for the development of regenerative therapies and for tissue engineering. Differentiation into dentinogenic cells was not explored as presently there are no specific odontogenic or dentinogenic genetic markers, apart from reparative dentine which is identified by bone-like markers (Chaussain et al. 2009; Dobie et al. 2002; Iohara et al. 2006).

As the DPSCs are from an easily accessible non-essential organ it may be possible to use the host's own progenitors for culture expansion and differentiation into required cell types for tissue regeneration. This includes differentiating into odontoblast-like cells for enhanced dentine regeneration after dental injury, but potentially into other mesenchymal tissue types such as bone, cartilage and adipose tissues as part of tissue healing and repair.

Future work may include examining the DPSC differential potential of different clone populations isolated from the same experiment or to compare against $P75^{+}$ cells. Determining if a different differentiation outcome is displayed by the different populations would further illustrate if there are differences in progenitor commitment and potential in the pulpal tissue. Variation of differential potential may assist in

determining the best isolation procedure of uncommitted and plastic DPSCs for use in tissue engineering.

It was previously discussed that neural crest stem cells were demonstrated to differentiate into mature cell types and were able to trans-differentiate into another cell type (section 1.6.2.1). As the DPSCs are derived from the neural crest they may display the differentiation instability seen with neural crest stem cells, where loss of the target cell phenotype may have drastic implications in regenerative therapy. Long-term culture and monitoring of terminally differentiated DPSCs may be a prudent method to verify if this occurs as this has not been previously investigated. Determining the progenitors with the most plastic differential potential and with a stable differentiation product is important for future DPSC selection, to select for the most immature populations for use in experimental studies and for the development of regenerative therapy.

With the establishment of a multipotent DPSC population the effects of DMP on specific aspects of DPSC behaviour were determined. The DMP components has previously shown enhanced therapeutic regenerative responses when supplemented into isolated DPSC cultures, producing an osteo-dentine matrix and forming a dentine-bridge during *in vivo* pulp-capping experiments (Begue-Kirn et al. 1992; Duque et al. 2006; Sloan and Smith 1999; Smith et al. 1994; Smith et al. 1990). This thesis illustrates that the addition of DMP enhanced DPSC numbers in a dose-dependant manner, as demonstrated by crystal violet staining (figure 4.4).

The DMP also maintained cell vitality as shown by its sustained metabolic activity and reduced caspase activity which may lead to cell apoptosis (figures 4.3 and 4.5). This demonstrated the regulatory effect of DMP that may prevent cell apoptosis, where the *in vivo* carious dentine environment is potentially a highly inflamed site which would otherwise promote cell death (Rutherford and Gu 2000). This makes DMP an attractive therapeutic component for dentine repair where the reduction of cell death and promotion of progenitor cell numbers may enhance dentine regeneration.

As part of dentine repair, the recruitment of DPSCs towards the dentine injury site is also an important aspect of tissue regeneration (Goldberg et al. 2009; Smith et al. 1994; Tecles et al. 2005). Collagen gels supplemented with DMP and cultured with DPSCs revealed a dose-dependant increase of cell numbers in the gels when compared with the unsupplemented gels (figure 4.11). A previous study on angiogenesis highlighted that the cocktail of growth factors in DMP may work synergistically with greater efficacy in directing cell behaviour (Zhang et al. 2011), which may explain the dose-dependant response shown by the cell migration experiments. However, the increase in cell numbers within the collagen gels could be due to an increase of cell migration into the gels, but may also be due to an increase of cell proliferation within the gels.

The use of time-lapse microscopy would greatly clarify the effects DMP has on DPSCs. If a monolayer culture was utilised, time-lapse imaging would provide evidence if DMP is a chemoattractant to the DPSCs where its migratory patterns could also be monitored. These results would build on the collagen gel cell migration

findings and clarify whether DMP increases DPSC migration, has a chemotactic effect or simply increases the DPSC numbers in the gels. Understanding the effects DMP has on DPSC migration may aid development of pulp capping strategies to enhance the natural reparative dentinogenesis process for regeneration of the injury site.

The study of DPSCs *in vitro* is not an ideal representation of the responses that occur *in vivo* and the development of methods to study DPSCs in the tooth environment would be more ideal. The isolated DPSCs were fluorescently stained, injected into a tooth slice and localised *in situ* 7 days post-injection (figure 5.8) as proof of concept that the cells are able to be localised when injected into tooth slices. This would allow a more valid approach to study cell behaviour when investigating dentine repair by replicating dentine injury in the tooth slice and following manipulation of the tooth slice environment.

Alternative methods to locate injected DPSCs would be to produce cells encoding fluorescent proteins such as green fluorescent protein (GFP). This can be achieved either by transfection of the GFP gene into the isolated DPSCs to create a stable line (Nakashima et al. 2002; Zheng et al. 2009) or to utilise GFP transgenic rodents and then isolate its DPSCs (Grottkau et al. 2010). However, fluorescent cells are shown to be prone to rapid photobleaching upon excitation making it difficult to study in long term experiments. Another disadvantage is the results can be affected by background autofluorescence from mineralised tissues (Pretty et al. 2002) which may cause interference to the fluorescent cells within the tooth slice.

A non-fluorescent method to overcome problems encountered with fluorescent cells and autofluorescence is the use of radiographic labelling of DPSCs by incorporation of tritiated thymidine (Fitzgerald et al. 1990; Messier and Leblond 1960; Tsiridis et al. 2009). This method may be used to monitor proliferation when injected into a tooth slice, although real time monitoring of cell behaviour is not feasible. This method require different samples to be fixed at various time points before sectioning for histological examination which would not clearly demonstrate cell migration when compared with time-lapse microscopy

An exciting cell labelling technology is to utilise luminescent, non-toxic, inorganic nanoparticles called quantum dots (QD) such as cadmium selenide QDs for cell labelling. These negatively-charged, water-soluble QDs are light-emitting nanoparticles that offer brighter fluorescence, more resistance to photobleaching and allow the use of multiple QDs compared with traditional fluorophores (Chan et al. 2002; Gao and Nie 2003).

This makes QDs favourable for *in vitro* or *in vivo* real-time long-term live cell imaging, ideal for monitoring cell processes in a tooth slice environment (Jaiswal et al. 2003; Ranjbarvaziri et al. 2011; Vibin et al. 2011). These QD fluorophores have broad excitation spectra but narrow emission spectra, so allowing many distinct QDs to be imaged at the same time, ideal for tracking several cells at once (Jaiswal and Simon 2004). The use of QD technology appears to be the most ideal candidate for cell tracking in future tooth slice experiments.

Once a cell staining/tracking method is determined, the use of a mechanical injection-microscope could be employed to provide a more consistent injection and minimise structural damage when injecting into a tooth slice. Used in combination with time-lapse microscopy to monitor DPSCs in the tooth slice, this developed methodology could be utilised for studying the progenitor cell behaviour *in situ*. Experiments could include replicating dentine injury to study DPSC migration in the tooth slice when injected into various locations of the slice, such as the pulp or dentine-pulp interface to determine if DPSCs are chemoattracted to the injury site.

Other tooth slice manipulation experiments could include the implant of bioactive molecules or materials such as growth factors (including TGF-β1, VEGF and PDGF), restorative materials or DMP (to build on chapter 4 cell migration results) and observing the migratory effects it has on the injected labelled DPSCs (Dobie et al. 2002; Sloan and Smith 1999). This would create a more valid approach in studying DPSC behaviour in response to extracellular factors encountered with dentine injury to further improve dentine regeneration therapy by understanding the factors that normally influence the DPSCs *in vivo*.

## 6.2. Conclusions

- Clonal isolation of DPSCs during early colony formation may select for the more immature fractions of progenitors in the pulp.

- Clonal populations of DPSCs are multipotent, where the same DPSC population demonstrated adipogenic, chondrogenic and osteogenic differentiation.

- DMP maintains DPSC vitality, increases cell numbers, reduces apoptosis and suggested to cause an enhanced migratory response.

- The bioactive molecules in DMP may have a synergistic function in causing an enhanced DPSC response.

- DPSCs can be fluorescently stained and localised when cultured in a tooth slice. A proof of concept that the cells can be followed *in situ* which opens possibilities for better study of cell behaviour during dentine injury and its response to tooth environment manipulation.

The uncommitted progenitor cells from the adult dental pulp show promise for tissue engineering with components of the dentine matrix demonstrating a regulatory role for dentine regeneration. In the future, these cells can potentially be harvested from the patients and manipulated for cell expansion and differentiation into patient-matched progenitor cells, or perhaps to utilise signalling factors of the dentine matrix for directing enhanced dental tissue regeneration.

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# Appendix

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Version1

06/03/2007

# Patient Information Sheet

## Effects of Dental materials on Dentine and Pulp

The School of Dentistry is planning to carry out a research project. You are invited to contribute to this project. Before you decide please take time to read the following information. You will have the opportunity to ask for more information if you wish to do so.

## What is the research about?

Tooth decay is a very common problem in most societies. Removal of decay and restoration (filling) of a decayed tooth is the usual treatment option. When a filling fails or the decay is too deep in the first place, more complex and expensive forms of treatment such as root canal treatment and crowns may be needed and if these treatments are not possible or fail, the tooth may need to be extracted.

Recent evidence has shown that certain dressing and filling materials used in dentistry can stimulate the cells in the centre of a tooth to produce extra layers of dentine. The dentine covers the delicate and sensitive cells in the centre of a tooth and gives the tooth the ability to protect itself from further damage. To an individual this can mean reduced pain and sensitivity following placement of fillings and possibly reducing the need for root canal treatment and extraction of teeth.

In our research we will look at many different materials commonly used for filling teeth and identify those with this positive effect. Results of this study will be published in scientific journals and could have significant effects on the way dental treatment is planned in the future.

## How can I contribute and what will happen if I take part

Your dentist has decided that you require a tooth (teeth) to be extracted. You could help us by giving permission for your extracted tooth to be used in this study. The extracted teeth will be anonymised and can not be traced back to you.

In the laboratory the teeth will be used to test the effects of different filling materials on the tooth substance including the cells within the pulp. These procedures **will not** involve any DNA testing. As the teeth are anonymised, we will not contact you after this.

## Do I have to take part?

You do not have to give your permission for your extracted tooth to be used in this study and it is entirely up to you to decide. However please be informed that the Hospital policy does not encourage patients to take away the extracted teeth for health and safety reasons. In the event that you do not wish to allow your extracted tooth to be used in this study, the Hospital will keep it for safe disposal.

If you wish to give your permission please sign the attached consent form.

## Contact details

If you feel that you need more information before giving your consent please contact:

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Version1
06/03/2007

# PARTICIPANT CONSENT FORM

**Title of Project:**

**Effects of Dental Materials on Dentine and tooth pulp**

Name of Researcher:

Leili Sadaghiani, Alan Gilmour, Alastair Sloan, Rachel Waddington

**Please initial box**

1. I confirm that I have read and understand the information sheet Version1 dated 06/03/2007 for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason. ☐
3. I agree to take part in the above study. ☐

| Name of Participant | Date | Signature |
|---|---|---|
| Name of Person taking consent (if different from researcher) | Date | Signature |