SECRETOMES FROM DENTAL-DERIVED MESENCHYMAL STEM CELLS FOR TISSUE REGENERATION

BY

FARHA ARIFFIN

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Department of Oral Biology

The School of Dentistry

College of Medical and Dental Sciences

University of Birmingham

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ABSTRACT

The role of secretome from dental-derived mesenchymal stem cell (MSC) as potential biological mediator for tissue regeneration has not been fully explored. Thus, this project aimed to evaluate this potential with the objectives of 1)to evaluate the effect of hypoxia incubation on stem cells proliferation and growth factors production and genes, 2) To evaluate the variabilities in the secretome production, 3) To explore the proteomic profiles of the secretomes and 4) To evaluate the effect of the collected secretome on pre-osteoblast cells proliferation, Alkaline Phosphatase (ALP) activity and migration. Rat bone marrow mesenchymal stem cells (BMSCs), periodontal ligament stem cells (PDLSCs) and dental pulp stem cells (DPSCs) were harvested and tested for stem cell markers, osteogenic and adipogenic differentiation, and various gene expression for growth factors and osteogenic potential. The cells were cultured in serum free media for 2 or 3 days and incubated in normal oxygen concentration (21%) or hypoxia (2%) before the conditioned media (CM) that contained the secretomes were collected. The CM were used for ELISA of VEGF, TGF-β1 and IGF-1, proteomic profiles via mass spectrometry and used in MTT, ALP and migration assays. PDLSCs have distinctive pattern of cell growth and lower osteogenic potential compared to BMSCs and DPSCs. The three stem cells produced VEGF and TGF-β1. IGF-1 was only detected in PDLSCs' CM. Hypoxia incubation promoted cell proliferation and increased VEGF and IGF-1 in the secretome. Different collection days and filtration of the CM could alter the protein content of secretome. Although proteomic profiles of PDLSC, DPSC and BMSC secretomes indicated diverse and complex mixtures of proteins, the effect of three sources of MSC secretomes produced almost similar effect on pre-osteoblast cell proliferation, ALP activity and cell migration. As a conclusion, secretomes of PDLSCs and DPSCs have a potential to be the biological mediators for tissue regeneration due to various protein content that are known important for tissue regeneration. Further research is needed to strengthen these findings.

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LIST OF ABBREVIATIONS

ORO	Oil Red O
ARS	alizarin red stain
ADSCs	adipose-derived stem cells
BMP	bone morphogenetic protein
BMSCs	bone marrow stem cells
BSP	bone sialoprotein
C/EBP-α	*CCAAT/ enhancer-binding protein alpha*
cbfa-1	*core binding factor alpha 1*
c-Fms	colony-stimulating factor-1 receptor
CM	conditioned media
DDIT4	DNA Damage Inducible Transcript 4
DFPCs	dental follicle progenitor cells
DPSCs	dental pulp stem cells
ECM	extracellular matrix
EGF	epidermal growth factor
FACB-4	*fatty acid binding protein 4*
FBS	fetal bovine serum
FGF	fibroblast growth factor
FOSB	*FBJ murine osteosarcoma viral oncogene homolog B*
GC-MS	gas chromatography mass spectrometry

GFP	green fluorescent protein
GSCs	gingival stem cells
GTR	guided tissue regeneration
ICAT	isotope coded affinity tags
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
LC-MS	liquid chromatography mass spectrometry
M-CSF	macrophage colony stimulating factor
MS	Mass spectrometry
MSC	mesenchymal stem cells
mTOR	mechanistic Target of Rapamycin
OCN	osteocalcin
OPG	osteoprotegrin
OSR2	*Odd-skipped related 2*
PBS	Phosphate Buffered Saline
PDL	periodontal ligament
PDLSC	periodontal ligament stem cells
PES	polyethersulfone
pO_2	partial pressure of oxygen
POSTN	*Periostin*
PPAR-γ	*perioxisome proliferator-activated receptor gamma*
PRP	platelet-rich plasma
RANKL	receptor activator of nuclear factor κB ligand

SCAP	stem cells from apical papilla
SFM	serum free media
SHED	stem cells from human exfoliated deciduous teeth
SILAC	stable isotope labelling by amino acids in cell culture
TGF-β1	transforming growth factor beta 1
TMT	Tandem Mass Tag
UMSC	umbilical cord stem cells
VCAN	*Versican*
VEGF	vascular endothelial growth factors
WNT2	*Wingless-type MMTV integration site family member 2*
α-MEM	alpha-modified minimum essential medium
RNA	ribonucleic acid
cDNA	complementary deoxyribonucleic acid
sqRT-PCR	semi-quantitative reverse transcriptase PCR
DNAase I	Deoxyribonuclease I
ELISA	enzyme-linked immunosorbent assay
OD	optical density
PANTHER	Protein annotation through evolutionary relationship

CHAPTER 1

1 INTRODUCTION

1.1 Periodontal Tissue Regeneration

Periodontal tissue regeneration is a complex process which not only involves alveolar bone but other complex structures such as periodontal ligament fibres and cementum as these structures are usually diminished during periodontitis. Periodontitis is the chronic inflammation of the periodontium which is multifactorial and characterized by the destruction of the periodontal tissues (Papapanou, Sanz et al. 2018). The destruction that occurs is not only due to bacterial infections from dental biofilm but part of it is also due to overt inflammation by the host response.

Most cases of healing after periodontal therapy is repair and not regeneration of the periodontal structures. Repair is a process in which the healing occurred but the architecture of the periodontium do not restore completely where as the tissues and function was totally renewed in periodontal regeneration (Schüpbach, Gaberthüel et al. 1993). Periodontal wound healing is considered complex process because not just involves various periodontal structures, the healing must occur on hard, avascular and non-vital hard tissue on the root surface.

Most critical part in regeneration of periodontal structures is the formation of properly aligned periodontal fibres which attach the cementum of the tooth surface at one end, to the alveolar bone proper at the other end. However, at the same time the regeneration of the alveolar bone vertically and horizontally must occur. There are many studies and case reports that have applied guided tissue regeneration (GTR) as an approach to regenerate the lost periodontal structures (Gottlow, Nyman et al. 1986, Nyman, Gottlow et al. 1987) and these have shown that the generated periodontal structures could be maintained up to 5 years (Gottlow, Nyman et al. 1992). The concept in GTR is to exclude the fast-growing cells ie; epithelial cells from migrating into the treated periodontal defect during post-operative healing by using periodontal membrane. This would enable the slow growing cells such as the osteoblasts and fibroblast to form the alveolar bone and periodontal ligament fibres (Gottlow, Nyman et al. 1984). However, GTR is technique sensitive as the manipulation of the

membrane need delicate handwork during surgical procedures and the membrane should be properly covered by the periodontal flaps, as if it is not, the procedure will likely be unsuccessful.

Most of the resulted periodontal wound healing predominantly involves bone formation without the formation of the newly regenerated periodontal ligament fibers inserted into the cementum (Palmer and Cortellini 2008). GTR provides an ideal model for tisue regeneration but the clinical result is unpredictable (Bartold, Gronthos et al. 2016). Consequently, over recent years, research have moved towards the usage of stem cells in periodontal regeneration (Iwata, Yamato et al. 2010, Monnouchi, Maeda et al. 2015, Torii, Konishi et al. 2015).

1.2 Bone remodelling

An important component in periodontal tissue regeneration involves alveolar bone formation. Osteoblasts are responsible for bone formation whereas osteoclasts are specialised cells involved in bone resorption. Osteocytes are mature osteoblasts embedded in the bone matrix. The precursor cells for osteoblasts are mesenchymal stem cells (MSC) (Huang, Yang et al. 2007) however the precursor cell for osteoclasts is monocytic-like which are derived from hemopoietic stem cells (Yamane, Kunisada et al. 1997). Bone formation and bone resorption occur dynamically during bone remodelling which involves the interaction between osteoblasts and precursors of osteoclasts. Bone remodelling is a continuous process and the process started with pre-osteoclast migration onto the bone surface, differentiated to multi-nuclear osteoclast and start the resorption process to remove the old mature bone. Then, the area will be covered by mononuclear cells which prepared the area and give signals for pre-osteoblast cells differentiation. This process is termed the reversal phase and it is followed with new bone deposition by the osteoblast (Hadjidakis and Androulakis 2006).

Osteoblasts are important in signalling osteoclastic bone resorption. Osteoblasts produce two molecules key to this process, which include macrophage colony stimulating factor (M-CSF-/CSF1) and receptor activator of nuclear factor κB ligand (RANKL). M-CSF is continuously produced by the osteoblast apart of fibroblast, keratinocytes and myocytes (Stanley 1998). However, RANKL is

generated when there are appropriate signals from certain key hormones and cytokines such as 1.25 vitamin D3, parathyroid hormone, prostaglandin E2, and interleukin-1 (IL-1), as well as bacterial components such as lipopolysacharides (Koide, Kinugawa et al. 2010). RANKL generated by the osteoblast subsequently binds to its receptor (RANK) on pre-osteoclasts. The binding of RANKL-RANK leads to differentiation of the pre-osteoclast to an osteoclast with the presence of MCS-F which also binds to it receptor on the pre-osteoclast which is the colony-stimulating factor-1 receptor (c-Fms) (Figure 1.1) (Koide, Kinugawa et al. 2010). Osteoblasts also produce another molecule, which is osteoprotegrin (OPG), and this has a similar structure as RANKL and will bind to RANK. This molecule will inhibit RANK-RANKL binding and stop the pre-osteoclast differentiating into an osteoclast. (Väänänen 2005).

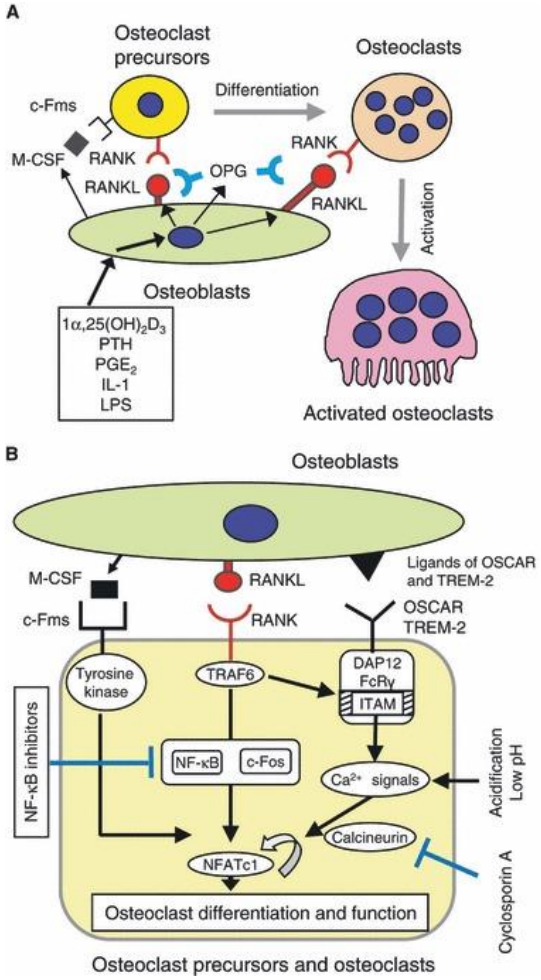


Figure 1.1 Interaction between RANK and RANKL

A. Interaction between RANK on the pre-osteoclast with RANKL on the osteoblast. B. Intercellular signals in the pre-osteoclast with interaction between RANKL and RANK; and M-CSF with c-Fms. RANK, receptor activator of nuclear factor κB; RANKL, receptor activator of nuclear factor κB ligand. Image reproduced from Koide et al, 2010.

Osteogenic differentiation occurred when the pre-osteoblast differentiates to the osteoblast for bone formation. The differentiation requires the expression of the *transcription factor core binding factor 1* (CBFA1/Runx2) and *osterix* genes (Hughes Francis J. 2006) by the pre-osteoblast. Certain growth factors are also required for this process to occur such as transforming growth factor β (TGF-β), bone morphogenetic protein (BMP), fibroblast growth factor (FGF), insulin growth factor (IGF), Wnt signalling molecules, and sonic hedgehog (Hughes Francis J. 2006).

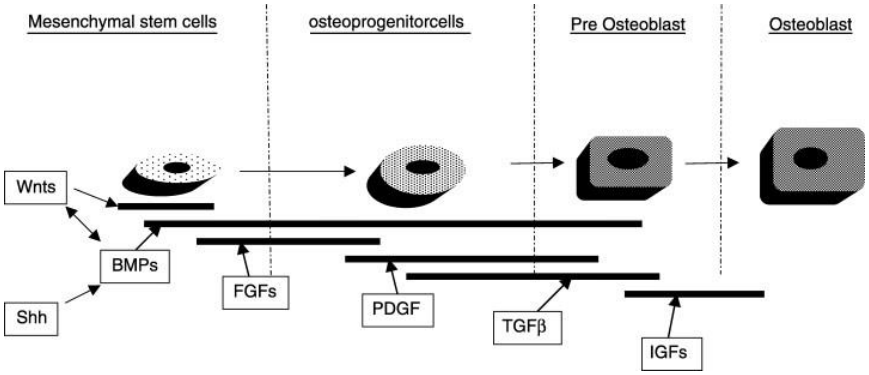


Figure 1.2 Growth factors and proteins in MSC differentiation
Image was reproduced from Hughes et al, 2006.

Osteogenic differentiation occurs in three stages starting with cell proliferation, followed by differentiation and finally matrix production and mineralization. During osteoblast differentiation,there are also associated increases in the production of alkaline phosphatase (ALP), collagen type I and other osteogenic differentiation markers such as osteocalcin (OC), bone sialoprotein (BSP) (Vater, Kasten et al. 2011). Markers that have been associated with osteoblast-lineage precursor cells include STRO-1, Runx2 and ALP. Other growth factors that have been previously mentioned such as BMP, FGF, and TGF-β are also useful in monitoring the osteogenic differentiation process. Notably osteoblastic differentiation can be achieved in *in vitro* cell cultures using osteogenic progenitors by standard cell culture supplemented with β-glycerophosphate and ascorbic acid for up to 3 weeks.

1.3 Mesenchymal Stem Cells

Stem cells are any undifferentiated cells that are able to differentiate into a range of specialized cell types. There are three types of differentiation potentials of the stem cells, which are totipotent, pluripotent and multipotent. Definition of totipotent is ability to differentiate into any cell types such as cells of morula within the first few days after fertilization; pluripotent is the ability to differentiate into several cell types of all three germ layers (ectoderm, mesoderm and endoderm) but not the whole organism; and multipotent is the ability to differentiate to a limited range of cells and tissues appropriate to their location (Bongso and Richards 2004). Two types of stem cells based on the

origins, are embryonic stem cells and adult stem cells. However, adult stem cells are easier to isolate for research purposes rather than embryonic stem cells.

Mesenchymal stem cells (MSCs) are undifferentiated multipotent cells that are capable of differentiating into various mesoderm cell lineages including osteogenic, adipogenic and chondrogenic lines. Some reports have suggested that MSC can also be differentiated to muscle cells and non-mesenchymal cells such as neuronal cells (Lavoie and Rosu-Myles 2013). They are present in almost any adult tissue such as bone marrow, umbilical cord and adipose tissue.

Several MSCs have been described including bone marrow stem cells (BMSC) (Friedenstein 1970), adipose-derived stem cells (ADSCs), dental pulp-derived stem cells (DPSCs) (Gronthos, Mankani et al. 2000, Gronthos, Brahim et al. 2002) and periodontal ligament stem cells (PDLSCs) (Melcher 1970, Aukhil, Simpson et al. 1986). There are other sources for MSCs which are dentally related including stem cells from human exfoliated deciduous teeth (SHED) (Miura, Gronthos et al. 2003), stem cells from apical papilla (SCAP) (Sonoyama, Liu et al. 2008), dental follicle progenitor cells (DFPCs) (Morsczeck, Gotz et al. 2005) and gingival stem cells (GSCs)(Zhang, Shi et al. 2009).

Since MSC have been reported in many tissues in the body, it has been proposed that these cells should fulfil the criteria outlined by the International Society for Cellular Therapy (ISCT). MSCs must be able to adhere to plastic material under standard culture conditions. They must also express three important markers which are CD105, CD73 and CD90. MSCs must not express CD45 (pan-leukocyte), CD34 (hematopoietic and endothelial cells), CD14/CD11b (monocyte/macrophage), CD79a/CD19 (B cells) and HLA-DR surface markers. Further criterion include the ability to differentiate to adipocytes, chondrocytes and osteoblasts when cultured *in vitro* (Dominici, Le Blanc et al. 2006). Other than that, more stringent characteristics of MSC has been suggested such as the ability of the isolated cells to regenerate their tissue of origin when implanted *in vivo* and the MSC must be able to show their self-renewal capacity although being transplanted multiple times *in vivo* (Bartold and Gronthos 2017).

Previously, BMSC were identified as colony-forming multipotent cells (Friedenstein 1970). These osteogenic progenitor cells were distinctive from the haematopoietic cells in bone marrow. BMSCs were able to adhere to tissue culture polystyrene and exhibited a fibroblast-like morphology (Friedenstein 1970). BMSCs are surrounded by minimal extracellular matrix (ECM) and this make these cells relatively more easily isolated compared with cells (DPSCs) which require the use of digestive enzymes to breakdown their associated ECM (Bianco, Riminucci et al. 2001).

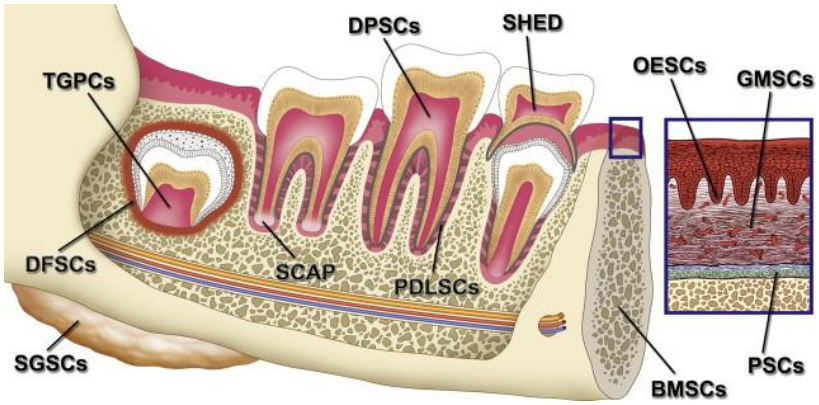


Figure 1.3 Dental Stem Cells

Schematic diagram of different time of dental stem cells. Bone marrow stem cells (BMSCs); Periodontal ligament stem cells (PDLSCs); Dental pulp stem cells (DPSCs); Stem cells from apical papilla (SCAP); Stem cells from human exfoliated deciduous (SHED); Tooth germ progenitor cells (TGPCs); Dental follicle stem cells (DFSCs); Salivary gland-derived stem cells (SGSCs); Oral epithelial stem cells (OESCs); gingival-derived MSCs (GMSCs), periosteum stem cells (PSCs). Source: (Egusa, Sonoyama et al. 2012)

Notably DPSCs were shown to be highly proliferative, possess a multi-differentiation potential and are able to form dentine-like tissue that contain pre-odontoblasts both *in vitro* and *in vivo* (Gronthos, Mankani et al. 2000, Gronthos, Brahim et al. 2002)..

In 2004, periodontal ligament stem cells (PDLSCs) were shown to differentiate to cementoblast-like cells, adipocytes and collagen-forming cells although PDL progenitor cells have been recognized long time ago since early 1970s (Melcher 1970, Aukhil, Simpson et al. 1986). PDLSCs express stem cell markers including STRO-1 and CD146/MUC18 (Seo, Miura et al. 2004).

1.4 MSCs in Tissue Regeneration

MSCs have extensively studied for tissue regenerations, for example in cases of myocardial infarction (Chen, Fang et al. 2004, Janssens, Dubois et al. 2006) and liver cirrhosis (Spahr, Chalandon et al. 2013). For example, intracoronary injection of autologous BMSCs in 34 patients with myocardial infarction showed better cardiac perfusion and function after 3 months compared to the control group which was injected with standard saline intracoronally (Chen, Fang et al. 2004).

In order for MSCs to offer clinical efficiency, they must be able to localise to the injured or damaged tissue potentially via either direct delivery or by migration down chemotactic gradients generated by certain signals produced at the injured site. Furthermore, the MSCs able to differentiate to cells relevant for repair of the desired tissue in the presence of appropriate signalling molecules. MSCs also may modulate the immune cells of the host to decrease levels of inflammation. This is relevant in clinical setting as it could reduce the inflammation of the post-operative healing (Liechty, Mackenzie et al. 2000, Jones and McTaggart 2008, Popp, Eggenhofer et al. 2008). Notably MSCs have been reported to suppress the immune response by modulating the action of T-cells, B-cells and dendritic cells (Liechty, Mackenzie et al. 2000, Jones and McTaggart 2008, Popp, Eggenhofer et al. 2008).

MSCs are relatively easily isolated, cultured and expanded *in vitro*. Most of the clinical trials using MSCs in humans are at relatively early phases (Wang, Qu et al. 2012, Spahr, Chalandon et al. 2013). MSCs in dental regenerative therapy has been reported in one case report in 2006 when autologous BMSCs were implanted into a periodontal infrabony defect and there was evidence of bone formation through periodontal examination and radiographic evaluation (Yamada, Ueda et al. 2006). However, in this case report, they implanted the BMSCs together with platelet-rich plasma (PRP) which is also known as a biologic mediator for bone regeneration on its own (Marx, Carlson et al. 1998). In one animal *in vivo* study, the implanted BMSCs in periodontal defect showed evidence of new cementum formation with functional periodontal ligament fibers (Yang, Rossi et al. 2010).

Although BMSCs are able to form bone in vitro and in vivo, BMSCs are relatively difficult to harvest as the procedure is invasive. Thus, alternative stem cells sources which are more easily harvested using non-invasive procedures are needed. In early 2000, Gronthos and his team have shown that DPSCs showed similar immunophenotype (Gronthos, Mankani et al. 2000) and multidifferentiation properties as BMSCs (Gronthos, Brahim et al. 2002).

In earlier *in vivo* studied, human DPSCs were implanted subcutaneously in rats using a collagen scaffold. After 2 months of implantation, histological slides showed evidence of bone formation (Graziano, d'Aquino et al. 2008). Autologous dog DPSCs were tested *in vivo* and implanted in intraosseous defect of dogs together with bovine bone graft (BioOss®). The grafted area with DPSCs showed thicker cementum and periodontal ligament fibers compared to the control sites which were grafted with BioOss® bone graft only. Furthermore, the apical migration of the junctional epithelium, which is common in healing after periodontal surgery and could disturb the regeneration, was restricted in the test group compared to the control group (Khorsand, Eslaminejad et al. 2013).

Recently, one randomized control clinical trial involving 29 patients with intrabony defects using DPSCs micrograft together with collagen sponge as a scaffold has been conducted and the result showed better probing pocket depth reduction and gained in clinical attachment after 12 months follow-up compared to control group which was grafted with collagen sponge only (Ferrarotti, Romano et al. 2018). However, the protocol in isolating DPSCs in their study was different. The autologous pulp was minced and processed in the tissue dissagregator machine immediately after tooth extraction, and the products of tissue disaggregation was combined together with the scaffold before implanted into the periodontal defects (Ferrarotti, Romano et al. 2018). There was no cell expansion involved *ex vivo* and no stem cell marker identification performed in this study, thus the stemness of the cells used was uncertain.

PDLSCs represent another dental stem cell which is easily harvested by gently extracting the tooth, enzymatically digested the PDL before the single cells suspension of cells were obtained. The PDLSCs of a swine showed promising results with evidence of cementum and organized new

sharpey's fibers formation when implanted in surgically-created periodontal defects (Liu, Zheng et al. 2008). In another study, when the PDLSCs were used with gelatin sponges (Gelfoam®) scaffold in periodontal defect of ovine, the cementum formation and Sharpey's fiber attachment were better than the negative control groups (Mrozik, Wada et al. 2013). In fact, one study claimed that autologous PDLSCs showed better cementum and PDL formations when compared with DPSCs in advance periodontitis models of beagle dogs (Park, Jeon et al. 2011).

Human autologous PDL progenitor cells from extracted teeth was then used in case report and the cells were expanded *in vivo* before being applied in the periodontal defect together with a hydroxyapatite scaffold (Feng, Akiyama et al. 2010). Results of this study showed positive periodontal improvements with reduction in probing pocket depth and reduced tooth mobility. The progenitor cells used were shown to have similar characteristics to those of PDLSCs.

Recent randomized control trial using PDLSC sheet together with BioOss® scaffold in 15 patients with intrabony periodontal defects showed that these cells were safe to be used in patients and did not caused any adverse effects. There was no increase in autoantibodies detected in blood profiles of patients post-operatively. However, this trial also showed that there was no added benefit of using a PDLSCs-based therapy for periodontal regeneration as similar bone gain was also achieved in control group that used only bone graft (Chen, Gao et al. 2016).

1.5 Limitations of MSCs as cell therapy

Few shortcomings of clinical applications of MSCs have beed discussed. Firstly, there is possibility that MSC may form ectopic tissue (Kim, Choi et al. 2013). Secondly, application of MSCs in the clinical setting has a number of challenges as the isolated cells need an appropriate storage, transportation and handling as their viability can be affected due to changes in the environment. Notable, the stem cells may not survive as injected cells and can be lost in the circulation especially by clearance in the liver and lung (Kim, Choi et al. 2013).

There are also differences of the environment during *in vitro* cell expansion compared with the microenvironment *in vivo*. One of the possible difference is oxygen concentration as different tissues environments have different oxygen concentrations (Ma, Grayson et al. 2009). Furthermore the presence of animal serum such as Fetal Bovine Serum (FBS) during cell culture could be another reason for failure of the implanted cells *in vivo* as the serum from different species could elicit immune reaction (Mackensen, Dräger et al. 2000, Jeffrey, Carl et al. 2004).

Evidence has shown that positive results by the implanted stem cells was not due to the ability of the cells to differentiate to the desired tissue but merely due to the paracrine effect of the cells (Wu, Chen et al. 2007, Gandia, Arminan et al. 2008, Kerkis, Ambrosio et al. 2008). Indeed, a recent study reported increased blood vessel formation when BMSCs were implanted in wound healing model compared with fibroblasts. However the BMSCs were not found at the walls of the newly-formed blood vessels but adjacent to vasculature area indicating that formation of the new blood vessels were not because of the differentiation of BMSCs but due to production of certain growth factors by the cells influencing the cellular response in the surrounding area (Wu, Chen et al. 2007).

In another study, human DPSCs which were labelled with green fluorescent protein (GFP) have been injected intramyocardially in myocardial infarction model of rats. After 4 weeks, the heart condition of the rats was reported to be improved with reduction in infact size and cardiac function. However, immunohistochemistry evidence showed that the DPSC were still present and maintained their proliferation rates. The findings suggested that the DPSCs did not differentiated into the cardiomyocytes but the positive cardiac response were believed to be the paracrine effect of the DPSCs (Gandia, Arminan et al. 2008).

In periodontal regeneration, latest animal study have showed no added benefit of the cell therapy using the PDLSCs from Beagle dogs, transplanted together with collagen bone graft in periodontal defects compared with using bone graft alone (Nuñez, Sanchez et al. 2018). However, when the MSCs were used in infrabony defects, positive results were obtained via possible paracrine effects from the MSCs (Nuñez, Sanz-Blasco et al. 2012, Suaid, Ribeiro et al. 2012) as they couldn't determine that the cells used remained active during healing period and were involved in the regeneration (Nuñez, Sanz-Blasco et al. 2012) and suggested the possibility of paracrine effect from the MSCs.

1.6 Secretomes

Currently, there are many studies focusing on the bioactive molecules produced by MSC as therapeutic agents rather than utilising the MSC itself as these paracrine factors released by the cells provide the mechanism of action by the transplanted cells rather than cells differentiation process (Chen, Tredget et al. 2008, Lee, Shah et al. 2010, Linero and Chaparro 2014, Mead, Logan et al. 2014, Paschalidis, Bakopoulou et al. 2014). The secretome is defined as the entirety of secreted products by a cell containing many types of growth factors, cytokines, enzymes, exosomes, microRNA and other soluble mediators (Haynesworth, Baber et al. 1996, Volmer, Stühler et al. 2005, Park, Bae et al. 2015). A sub-component of the secretomes are microvesicles including exosomes which are vesicles sized 50-100nm in diameter originating from microvesicular bodies that undergo exocytosis from the cell (György, Szabó et al. 2011).

MSCs secretome studies have used various tissue sources such as from umbilical cord stem cells (UMSC) (Wang, Xu et al. 2015) , ADSC (Alvarez-Llamas, Szalowska et al. 2007, Lee, Jin et al. 2012, Curtis, Hannett et al. 2018) but most of the studies were using BMSCs (Hung, Pochampally et al. 2007, Chen, Tredget et al. 2008, Osugi, Katagiri et al. 2012, Ranganath, Levy et al. 2012, Ando, Matsubara et al. 2014, Maffioli, Nonnis et al. 2017, Ogata, Matsumura et al. 2018).

Earlier studies on the effect of secretomes of stem cells involved research on wound healing (Chen, Tredget et al. 2008). When the secretomes from BMSCs were compared with the secretomes from dermal fibroblasts, it was found that the migration of macrophages, endothelial cells and keratinocytes were enhanced compared with the secretomes from dermal fibroblast. This effect was probably due to higher levels of growth factors such as vascular endothelial growth factor (VEGF), insulin-like growth factor 1(IGF-1), epidermal growth factor (EGF), angiopoietin-1 and stromal derived factor-1 in BMSCs secretomes that are important in stimulating wound healing (Chen, Tredget et al. 2008).

In a further study using an *in vitro* wound healing model involving ADSCs, similar findings were obtained whereby the culture medium of ADSCs enhanced proliferation of keratinocytes and dermal fibroblasts (Lee, Jin et al. 2012). Besides wound healing, secretome of MSCs have been studied for healing of the cardiac tissues following myocardial infarction (Timmers, Lim et al. 2011, Ranganath, Levy et al. 2012) and bone healing (Osugi, Katagiri et al. 2012, Ando, Matsubara et al. 2014, Ogata, Katagiri et al. 2015).

The effects of secretomes from BMSCs on bone regeneration has been analysed in relatively few studies and most of these studies have shown positive stimulatory effects. CM from cultured BMSCs were tested on other BMSCs either in *in vitro* or *in vi*vo animial studies. In 2012, a study from Japan explored the effect of secretome of human BMSCs in bone regeneration. The CM that contained the secretomes were added to rat BMSCs in culture and the level of osteogenic markers of the cultured cells were subsequently analysed and compared with unsupplemented control cell culture. It was found that the level of *osteocalcin* (OCN) and *Runx2* genes were expressed higher in the secretome supplemented cultures (Osugi, Katagiri et al. 2012). Furthermore, in the same study; the BMSC CM were used together with the BMSC in a hydrogel delivery system to regenerate bone defect in rat's calvaria. It was found that the bone regeneration rate of the damaged calvaria was significantly higher when the BMSCs were added with secretome compared with the control group in which only live BMSC were used (Osugi, Katagiri et al. 2012).

These findings were similar in another study which utilised a distraction osteogenesis model in rats. In this study, human BMSCs were cultured and the collected media was tested in Alkaline Phosphatase (ALP) and osteoblast differentiation assays in mice. The CM were injected trancutaneously into the centre of the distraction osteogenesis site together with a collagen matrix gel and formation of new bone was increased in the presence of the secretomes (Ando, Matsubara et al. 2014). Secretome studies using CM from BMSCs is as listed in Table 1.1.

	Tissue regeneration involved:
Chen et al, 2008	Wound healing
Hung et al, 2007	Angiogenesis
Osugi et al, 2012	Bone regeneration
Tsuchiya et al, 2013	Implant osteointegration
Ando et al, 2014	Bone regeneration
Ogata et al, 2015	Bone regeneration
Sharabi et al, 2016	Pulpal reaction
Xu et al, 2016	Bone regeneration
Inukai et al, 2016	Periodontal regeneration

Table 1-1 List of studies using BMSC secretomes

BMSC secretomes have been used and most of the studies listed involved in different types of tissue regeneration.

Secretomes from other MSCs have also been tested for bone regeneration models. Human AdMSCs CM were added to human blood plasma hydrogels which acted as scaffolds in a rabbit's mandibular bone defect model. The study found that AdMSCs CM which contained the secretomes was able to regenerate bone to a similar extent as to when the stem cells alone were used. These data indicated that the secretomes alone were able to induce bone formation without the need for live cells (Linero and Chaparro 2014).

1.7 Secretomes from Dental Stem Cells

Secretome of dental stem cells have been tested for tissue regeneration in *in vitro* and *in vivo* studies such as nerve (Yalvaç, Yarat et al. 2013, Mead, Logan et al. 2014), lung (Wakayama, Hashimoto et al. 2015) heart (Yamaguchi, Shibata et al. 2015) and bone injuries (Park, Bae et al. 2015).

A recent in vitro study using the secretome of MSCs from dental tissue was able to show evidence of osteogenic differentiation. The study cultured human dental pulp cells and tested the collected secretomes on osteogenic differentiation of dental follicle cells. Furthermore, the authors reported on the expression of several genes important in osteogenic differentiation such as *Wingless-type MMTV integration site family member 2* (*WNT2*), *Versican* (*VCAN*), *Odd-skipped related 2* (*OSR2*), *FBJ murine osteosarcoma viral oncogene homolog B* (*FOSB*) and *Periostin* (*POSTN*) which were up-regulated when dental follicle cells were cultured with the secretomes from DPSCs (Park, Bae et al. 2015).

However, one recent *in vitro* study failed to show mineralization induction using an osteogenic assay when the conditioned medium of dental pulp were used (Fujio, Xing et al. 2015). However, the researchers did identify bone regeneration induction in an *in vivo* distraction osteogenesis mouse model when using similar CM. The researchers speculated that the role of the CM was mainly in promoting angiogenesis at the healing area rather than promoting new bone formation (Fujio, Xing et al. 2015).

Studies on the secretome of PDLSCs were also emerging especially in the past 5 years (Rajan, Giacoppo et al. 2016, Ratajczak, Hilkens et al. 2016, Nagata, Iwasaki et al. 2017). In of the studies, the secretome of human PDLSC have been tested in vitro and in vivo for angiogenic potential using chorioallantoic membrane assay. Despite positive effect of the PDLSC CM on the proliferation and migration of the endothelial cells, the study failed to show any positive effect on blood vessels formation compared to positive control group which used the VEGF (Ratajczak, Hilkens et al. 2016).

The secretome of PDLSCs also have been shown to regenerate periodontal structures in animal models of the periodontal defect using rats (Nagata, Iwasaki et al. 2017, Qiu, Wang et al. 2020) There was an evidence of cementum-like structures with inserting periodontal fibres 4 weeks after implantation of PDLSC CM that contained the secretome with collagen membrane.

Another interesting study tested human PDLSC CM in mouse model of multiple sclerosis in which the PDLSC CM were injected into the spinal cord of the mouse. Results of the study showed regression of the disease progression together with evidence of spinal cord remyelination (Rajan, Giacoppo et al. 2016).

	Secretome of:	**Experiments involved:**
Aranha et al, 2010	DPSC	Angiogenesis
Paschalidis et al, 2014	DPSC	Pulp repair
Mita et al, 2015	SHED	Nerve regeneration
Bakopoulou et al, 2015	SCAP	Angiogenesis
Nagata et al, 2016	PDLSC	Periodontal regeneration
Rajan et al, 2016	PDLSC	Immunosuppression
Ahmed et al, 2016	DPSC	Neural differentiation
Xia et al, 2016	PDLSC	Osteogenic differentiation
Kumar et al, 2017	DPSC, SCAP, DFSC	Neural differentiation
Kolar et al, 2017	DPSC, SCAP, DFSC	Nerve regeneration
Kumar et al, 2018	DPSC, SCAP, DFSC	Osteogenic differentiation

Table 1-2 List of secretome studies using dental stem cells
Dental-derived secretomes have been used and most of the studies listed involved in different types of tissue regeneration.

1.8 Variablity in Conditioned Medium (Secretome) Preparation

The secretome was generally obtained from the conditioned medium (CM) during cell culture after a range of culture durations. Despite many studies on the secretomes of MSCs, not all studies report on the exact content (growth factors and cytokines) of the secretomes (Chen, Tredget et al. 2008, Aranha, Zhang et al. 2010, Tsuchiya, Hara et al. 2013, Paschalidis, Bakopoulou et al. 2014, Al-Sharabi, Mustafa et al. 2016, Ratajczak, Hilkens et al. 2016). Many factors contribute to their differential effects such as the different origins of the MSCs (Osugi, Katagiri et al. 2012, Paschalidis, Bakopoulou et al. 2014, Ogata, Katagiri et al. 2015, Ratajczak, Hilkens et al. 2016), different oxygen concentration during tissue culture (Hung, Pochampally et al. 2007, Chen, Tredget et al. 2008, Fujio, Xing et al. 2015, Giacoppo, Thangavelu et al. 2017), different time points used for collecting the CM (Paschalidis, Bakopoulou et al. 2014, Tachida, Sakurai et al. 2015) and due to a range of different laboratory techniques used.

1.8.1 Oxygen Incubation

The oxygen concentration in the body varies from one tissue to another (Ivanovic 2009). One recent study has shown that the level of partial pressure of oxygen (pO_2) in the bone marrow environment is in the range of 11.7 to 31.7mmHg which equivalent to 1.5 to 4.2% (mean: 2.7%) (Spencer, Ferraro et al. 2014). A previous study also showed that slightly higher oxygen concentration which was 49mmHg (7%) (Kofoed, Sjøntoft et al. 1985). The partial pressure of dental pulp was reported to be around 23.2 mmHg (3.3%)(Yu, Boyd et al. 2002).

Currently, there is no study that measures the partial pressure of oxygen in periodontal ligament directly. However, one study in 1990 demonstrated that the oxidative state (which also indirectly relates to the level of oxygenation) was lower in periodontal ligament and dental pulp areas (Tuncay, Haselgrove et al. 1990).

When the PDLSCs were cultured in lower oxygen concentration, results for the cell proliferation were found to be inconsistent between studies (Choi, Jin et al. 2014, Zhou, Fan et al. 2014, Kawasaki,

Sumita et al. 2015). There are studies that showed PDLSCs favour hypoxia environment to stimulate proliferation (Amemiya, Matsuzaka et al. 2008, Choi, Jin et al. 2014, Zhang, Zhang et al. 2014) even up to 7 days, however, one study showed evidence of cell death and reduce number of cells after 24 hours exposure to lower oxygen compared with control cells that was incubated in 21% oxygen (Kawasaki, Sumita et al. 2015). There has also been a study that showed no significant difference in proliferation rate between hypoxic and normoxic cells (Zhou, Fan et al. 2014). However, result of the studies by Kawasaki and Zhou agreed that hypoxia environment increased the stemness of the PDLSCs as the expression of the pluripotency markers was reported higher compared with normoxia group (Zhou, Fan et al. 2014, Kawasaki, Sumita et al. 2015).

The effect of hypoxia environment on proliferation of DPSCs was also not conclusive. Two studies reported that there were no significant changes of DPSCs proliferation between hypoxia (2%-8% oxygen) and normoxia (21% oxygen) (Agata, Kagami et al. 2008, Zhou, Fan et al. 2014). However, there are also studies that reported DPSCs have higher proliferation rate in 1% to 5%. hypoxia for up to 7 and 14 days (Sakdee, White et al. 2009, Gong, Quan et al. 2010, Iida, Takeda-Kawaguchi et al. 2010, Ahmed, Murakami et al. 2016)

The concept of lower oxygen environment to maintain the stemness of the stem cells is becoming more recognised (D'Ippolito, Diabira et al. 2006, Agata, Kagami et al. 2008, Ma, Grayson et al. 2009, Ma, Grayson et al. 2009, Mohyeldin, Garzon-Muvdi et al. 2010, Choi, Jin et al. 2014, Zhou, Fan et al. 2014, Kawasaki, Sumita et al. 2015). In order to maintain the stemness in their niches, the MSC needs a few regulators and mechanism to prevent it from differentiating. DNA Damage Inducible Transcript 4 (DDIT4) is regulator that responsible to inhibit the mechanistic Target of Rapamycin (mTOR) pathway in which, once the pathway is activated, the MSC will start differentiating (Gharibi, Farzadi et al. 2014, Gharibi, Ghuman et al. 2016). Other than that, low oxygen environment (hypoxic environment) to the MSCs will also maintain the stemness of the MSC as it involves the upregulation of DDIT4 and HIF-1α (Gharibi, Ghuman et al. 2016).

However, most of secretome studies incubated their cells in ambient oxygen (~21% oxygen and 5% carbon dioxide) (Osugi, Katagiri et al. 2012, Inukai, Katagiri et al. 2013, Yalvaç, Yarat et al. 2013, Ando, Matsubara et al. 2014, Paschalidis, Bakopoulou et al. 2014, Kawai, Katagiri et al. 2015, Ogata, Katagiri et al. 2015, Ahmed, Murakami et al. 2016, Al-Sharabi, Mustafa et al. 2016, Xia, Tang et al. 2016, Kumar, Kumar et al. 2017). Indeed stem cells studies that have used hypoxic incubation for the secretome productions are very limited as listed in Table 1-3 (Hung, Pochampally et al. 2007, Chen, Tredget et al. 2008, Aranha, Zhang et al. 2010, Chang, Chio et al. 2013, Fujio, Xing et al. 2015, Ahmed, Murakami et al. 2016, Giacoppo, Thangavelu et al. 2017).

Study	Cells used	Oxygen concentration
Hung 2007	hBMSCs	1%
Chen 2008	hBMSCs	0.5%
Aranha 2010	hDPSCs	1%
Chang 2013	hBMSCs	0.5%
Fujio 2015	hDPSCs	1%
Ahmed 2016	hDPSCs	3%, 5%
Giacoppo 2017	hPDLSCs	3%

Table 1-3 List of secretome studies using hypoxia incubation and different oxygen concentration used

It has been shown that hypoxic incubation induced MSCs to produce higher levels of growth factors such as VEGF, TGF-B1 and IGF-1 (Linero and Chaparro 2014). Moreover CM collected under hypoxic conditions promoted greater level of angiogenesis than CM collected in normal incubation (Fujio, Xing et al. 2015). It may be possible that these findings were due to the deprivation of nutrition, and subsequently the stressed cells produced more ‘protective’ secretomes in order to create a conducive environment for their survival. In addition it may also be that less metabolic products were produced by the cells under the serum-deprived condition as it is well-known that increased metabolic by-products could cause cytotoxic effects (Paschalidis, Bakopoulou et al. 2014).

This study aims to address the effects of hypoxia on MSC proliferation, gene expression and secretome production.

1.8.2 Filtration of the CM

Most of the secretome studies have wide variability in collection of the CM especially in relation to filtration and centrifugation of the CM. Filtration of laboratory solutions have been used as sterilisation method as the membrane used in the filter could eliminate microorganisms and coarse particles. The usual pores sizes of the membrane, which commonly used in tissue culture, are 0.2 or 0.22µm. There are many types of membrane used such as cellulose acetate, cellulose nytrate, nylon, polyethersulfone (PES) or glass fibers. Each type of membrane has different characteristics in filtering the components of the solution. PES membrane is the most suitable for tissue culture as it has very low protein binding compared to other types of membrane. Many studies however do not mention the filtration of the CM (Chen, Tredget et al. 2008, Osugi, Katagiri et al. 2012, Inukai, Katagiri et al. 2013, Ando, Matsubara et al. 2014, Ahmed, Murakami et al. 2016, Katagiri, Osugi et al. 2016). However, there were few studies that filter the samples with 0.22µm membrane (Yalvaç, Yarat et al. 2013, Linero and Chaparro 2014, Paschalidis, Bakopoulou et al. 2014, Bakopoulou, Kritis et al. 2015).

None of the secretome studies so far compared and discussed the effect of the filtration in secretome content. Thus, this thesis aims to evaluate the effect of growth factors level between sterile-filtered and unfiltered CM.

1.8.3 Different Collection Days of the CMs

Other than the variables discussed above, different days of CM collection should also be taken into consideration in secretome studies. Most of the studies reported so far have collected the CM after 24 to 48 hours. The reasoning for this is to reduce the metabolic byproducts generated from the cells which could occur during serum deprivation in longer cultures which could also be harmful to the

other cell types (Paschalidis, Bakopoulou et al. 2014). There have been few studies that collect the CM for longer than 2 days and that are still able to show that the CMs were biologically active (Tachida, Sakurai et al. 2015, Wang, Xu et al. 2015). Nonetheless, whether the content of the growth factors in the secretome is higher in longer culture condition is not yet known. Thus, this study would like to evaluate the differences in growth factors production between short and long culture durations.

The variability in CM preparation for secretome production in various studies were simplified in Table 1-4.

Study	Cells	Passage	Collection day	Filtration	Incubation
Chen et al, 2008	hBMSCs	3	1	-	Normoxia Vs Hypoxia (0.5%)
Hung et al, 2007	hBMSCs	3-6	2	-	Normoxia Vs Hypoxia (1%)
Aranha et al, 2010	hDPSCs	Not mentioned	1	-	Normoxia vs Hypoxia (1%)
Osugi et al, 2012	hBMSCs	3-9	2	-	Normoxia
Kim et al, 2013	hBMSCs	6-10	1	0.2µm	Normoxia
Tsuchiya et al, 2013	rBMSCs	3	2	-	Normoxia
Chang et al, 2013	hBMSCs	3-7	1	-	Normoxia Vs Hypoxia (0.5%)
Inukai et al, 2013	hBMSCs	3-9	2	-	Normoxia
Paschalidis et al, 2014	HDPSCs	4-8	4,8,12,16,20,24	0.2µm	Normoxia
Ando et al, 2014	hBMSCs	Not mentioned	2	-	Normoxia
Kawai et al, 2015	hBMSCs	3-9	2	-	Normoxia
Wakayama et al, 2015	hBMSCs, hSHEDs	3-9	2	-	Normoxia
Fujio et al, 2015	hDPSCs	4-8	2	-	Normoxia vs Hypoxia (1%)
Mita et al, 2015	hSHEDs	3-5	2	-	Normoxia
Park et al, 2015	hDPSCs	Not mentioned	12 hours	0.2µm	Normoxia
Ogata et al, 2015	hMSCs	Not mentioned	2	-	Normoxia
Tachida et al, 2015	rBMSCs, rASCs, rDPSCs	Not mentioned	3	0.2µm	Normoxia
Wang et al, 2015	hUCM	Not mentioned	2	0.2µm	Normoxia
Ahmed et al, 2016 (a)	hDPSCs, hBMSCs	Not mentioned	1	-	Normoxia

Ahmed et al, 2016 (b)	hDPSCs	4-5	1	-	Normoxia vs Hypoxia (3% and 5%)
Kumar et al, 2016	hBMSCs, hDPSCs, hSCAPs, hDFSCs	3-7	2	0.2µm	Normoxia
Katagiri et al, 2016	hBMSCs	3	2	-	Normoxia
Sharabi et al, 2016	hBMSCs	3	2	-	Normoxia
Xu et al, 2016	hBMSCs	3-6	1	-	Normoxia
Ratajczak et al, 2016	hPDLSCs	3-8	1	-	Normoxia (preconditioned with Deforaxamine to mimic hypoxia)
Xia et al, 2016	hPDLSCs	Not mentioned	3	0.2µm	Normoxia
Nagata et al, 2016	hPDLSCs	2-6	2	0.2µm	Normoxia
Maffioli et al, 2017	hBMSCs	Not mentioned	18 hours	-	Normoxia
Giancoppo et al, 2017	hPDLSCs	2	1	-	Normoxia vs Hypoxia (3%)
Kumar et al, 2017	BMSCs, DPSCs, SCAPs, DFSCs	3-7	2	0.2µm	Normoxia
Kumar et al, 2018	BMSCs, DPSCs, SCAPs, DFSCs	3-7	2	0.2µm	Normoxia

Table 1-4 The variability in secretome collection and preparation

1.9 Growth Factors in Secretomes

Growth factor is a protein or biological mediator that stimulates or inhibits other cells' activities and behaviour. Indeed, for any biological mediators to be effective in tissue regeneration, it most likely must possess the ability to promote angiogenesis.

Vascular endothelial growth factor (VEGF) plays an important role in the formation of new blood vessels (Scheven, Man et al. 2009, Yalvaç, Yarat et al. 2013) by promoting endothelial cell proliferation, migration and differentiation (Ferrara 2001). VEGF has several subtypes such as VEGF-A, VEGF-B, VEGF-C and VEGF-D. VEGF-A will react with either VEGF receptor 1 (VEGFR-1) and VEGF receptor 2 (VEGFR-2) to regulate angiogenesis and blood vessel permeability. VEGF-B binds only to VEFGR-1. VEGF-C and VEGF-D on the other hand binds to VEGFR-3 which is located on the endothelial lympatic cells (Neufeld, Cohen et al. 1999, Takahashi and Shibuya 2005).

Transforming growth factor-β1 (TGF-β1) is a growth factor that plays an important role not only in proliferation of the MSCs but in differentiation of the cells and production of extracellular matrix (Melin, Joffre-Romeas et al. 2000, Tetsuro and Kohei 2009). In fact, there was report stating that the effect of TGF-β1 is more pronounced in cell differentiation compared with cell proliferation (He, Yu et al. 2008). When the DPSCs were co-cultured with TGF-β1, the cells showed evidence of odontoblastic differentiation by the increased ALP activity, formation of mineralized nodules, changes in cells morphology and expression of dentin sialoprotein and dentin matrix protein and the cells exert proliferation when co-cultured with Fibroblast Growth Factors (FGF) (He, Yu et al. 2008)

Insulin growth factor 1 (IGF-1) is also important in tissue growth and and regulates MSC migration and proliferation (Li, Yu et al. 2007). There are two types of IGF; IGF-1 and IGF-2 which are both small molecule polypeptides (Bendall, Stewart et al. 2007). Both IGFs are important in stem cell proliferation but IGF-1 is more prominent in adult stem cells. IGF-1 binds to its receptors, IGFR-1 and IGFR-2. The affinity of IGFR-1 is higher compared to IGFR-2. The IGFs binds to larger binding

protein (IGFBP) present in the systemic circulations to enable transportation to respective tissues. There are 7 types of binding proteins (IGFBP-1 to IGFBP-7) (Youssef, Aboalola et al. 2017). Apart from IGFBP-7, all other binding proteins have high affinity to IGFs (Kim, Rosenfeld et al. 1997). IGFBP 1, 2 and 4 are known to migrate through blood vessels and regulate cellular activities whereas IGPBP 3 cannot cross the blood vessels and therefore remain in the circulation. Other IGFBPs are present at very low concentrations and are considered not to have any significant impact to cellular activities (Kim, Rosenfeld et al. 1997). Once the proteolysis of the binding between IGF-IGFBPs complex occur, the IGF-1 will be free to interact with the IGFR-1 and start intracellular signals to increase cell proliferation (Kim, Rosenfeld et al. 1997). Apart from increasing cell proliferation, IGF-1 also have been showed to regulate the differentiation of the MSCs into osteoblasts (Cornish, Grey et al. 2004).

Thus, the effects of growth factors in secretomes from MSCs on bone regeneration has been studied and the growth factors specifically VEGF and IGF-1 have shown positive stimulatory effects to promote angiogenesis and osteogenesis (Osugi, Katagiri et al. 2012, Katagiri, Osugi et al. 2016, Sakaguchi, Katagiri et al. 2017)

1.10 Mass Spectrometry for Proteomic Profiling of the Secretome

The secretome content consists of complex mixture of proteins (Estrada, Li et al. 2009, Osugi, Katagiri et al. 2012, Kim, Choi et al. 2013, Ando, Matsubara et al. 2014, Paschalidis, Bakopoulou et al. 2014, Fujio, Xing et al. 2015, Kawai, Katagiri et al. 2015, Ogata, Katagiri et al. 2015, Tachida, Sakurai et al. 2015, Ahmed, Murakami et al. 2016, Al-Sharabi, Mustafa et al. 2016, Maffioli, Nonnis et al. 2017). For the past 10 years, secretome studies using mass spectrometry have emerged (Alvarez-Llamas, Szalowska et al. 2007, Sarojini, Estrada et al. 2008, Estrada, Li et al. 2009, Lee, Kim et al. 2010, Tasso, Gaetani et al. 2012, Tachida, Sakurai et al. 2015, Dou, Wu et al. 2017, Maffioli, Nonnis et al. 2017) for analysis of protein contents in the secretome.

Mass spectrometry (MS) is a technique whereby the proteins are identified via its molecular mass of the protein's peptides that were fragmented via ionization procedure. The ionized molecular mass of the fragmented peptides is then matched to the protein database based on their mass (*m*) over charged (*z*) ratio (*m/z*) to identify specific proteins. Liquid chromatography mass spectrometry (LC-MS) is one of the MS types apart from gas chromatography mass spectrometry (GC-MS) (Pitt 2009). LC-MS as the name suggest, can analyse the proteins in a liquid samples and has high specificity (Pitt 2009).

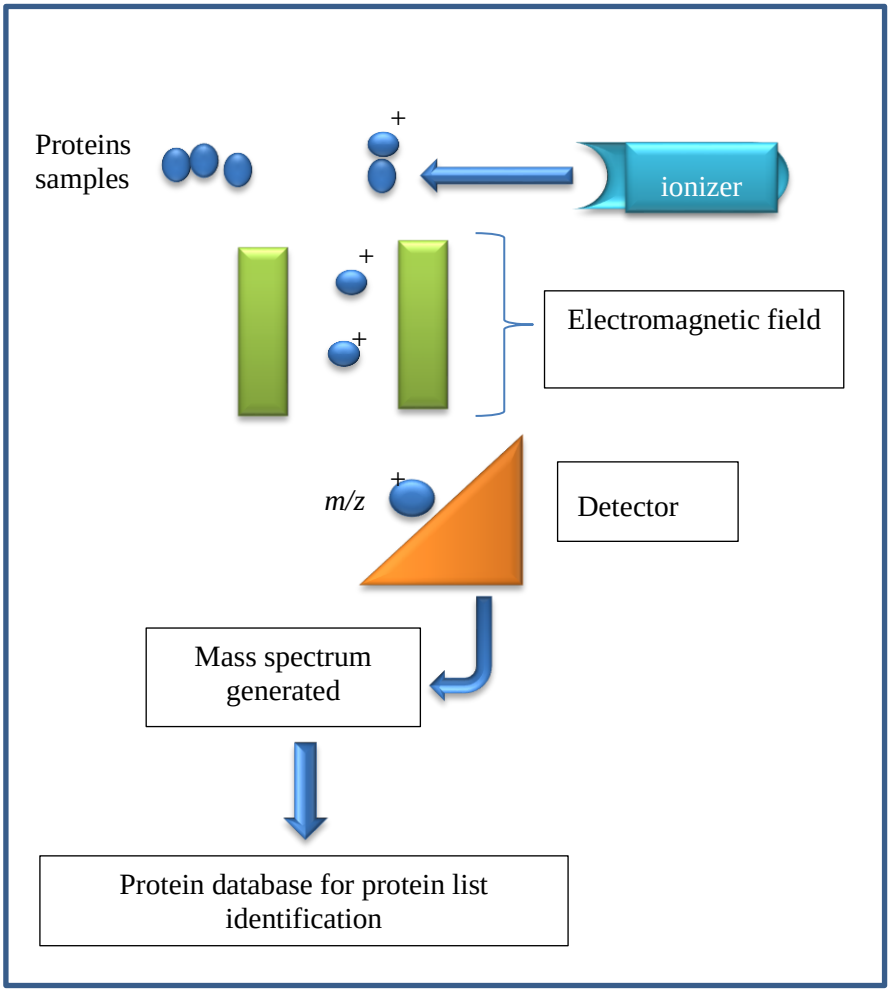


Figure 1.4 Simplified diagram of the MS process

Improvement in MS techniques involves relative quantification of the identified proteins of any samples in relation to the control sample. Quantitative mass spectrometry can be performed either via label-based or label-free or label-free techniques (Kupcova Skalnikova 2013). There are few types of quantified MS such as stable isotope labelling by amino acids in cell culture (SILAC) (Ong, Blagoev et al. 2002) or isotope coded affinity tags (ICAT) (Gygi, Rist et al. 1999). Tandem Mass Tag (TMT) is another method in quantitative MS (Zhang and Elias 2017) based on two pairs of tags peptides labelled to the sample peptides. The tags migrate together with the sample peptides during peptides separation and it does not interfere in *m/z* ratio detection of the samples as occurs normally for other labelling techniques (Thompson, Schäfer et al. 2003).

Proteomic profiles of BMSC and DPSC secretomes using mass spectrometry analysis have been reported in a few studies (Tachida, Sakurai et al. 2015, Kumar, Kumar et al. 2018) and one recent study compared BMSCs and SCAPs (Shi, Yuming et al. 2016). However, until recently data on PDLSC secretome is not yet available although the proteomic profiles of cellular PDLSCs has been published in two studies (Mrozik, Zilm et al. 2010, Eleuterio, Trubiani et al. 2013).

One label-based mass spectrometry data using TMT involving secretome of dental MSCs have been performed (Shi, Yuming et al. 2016). The secretomes of BMSCs were compared with secretomes of SCAPs and they found that 35% of the total proteins were secreted at higher level by the SCAPs. However, SCAPs have lower secretion of pro-angiogenic proteins compared to BMSCs. However, SCAPs have lower secretion of pro-angiogenic proteins compared to BMSCs. Further quantification mass spectrometry data is available in the study by Tachida which compared the proteomic profiles of BMSCs, DPSCs and ADSCs secretomes (Tachida, Sakurai et al. 2015). The study used label-free quantification as they analysed the signal intensity of the identified protein and normalised to internal control. As proteomic data on the dental secretomes was still limited, this study included analysis of the secretomes of the PDLSCs and DPSCs using MS.

Thus, this PhD study focused on the secretomes from PDLSCs and DPSCs in comparison to widely studied BMSCs secretome specifically related to the effect of hypoxia incubation, different

collection days of the CM and filtration of the CM to the secretome content. Furthermore, the effect of the secretome in the BMSCs, PDLSCs and DPSCs CM to the other cell's bioactivity were also assessed.

1.11 Project Aims

1.12 Research Questions:

i. What are the effects of hypoxia incubation, different collection day and filtration of the CM on the secretome content from the BMSCs, PDLSCs and DPSCs cultures?

ii. What are the protein or growth factors profiles contained in secretomes from bone marrow, dental pulp and periodontal ligament MSCs?

iii. What are the effects of the secretome from bone marrow, dental pulp and periodontal ligaments on cells viability, cells migration and mineralisation potential of pre-osteoblast cells which one of the components in tissue regeneration?

1.13 Aim of the Research:

It is hypothesized that secretomes from dental MSCs will have positive effect on cell growth, osteogenic potential and migration ability which could lead to improvement in tissue regeneration. The aim of this PhD study is to investigate the role of the PDLSCs' and DPSCs' secretomes in comparison to secretome of BMSCs on therapeutic potential for tissue regeneration. In order to meet this aim, several research objectives were addressed which included:

i. To evaluate the characteristics of BMSCs, PDLSCs and DPSCs in terms of the gene expression for stem cell markers, growth factors and osteogenic markers. In all aspects of this study, BMSCs were used as the control cells compared to PDLSCs and DPSCs.

ii. To evaluate the effect of hypoxia incubation on the proliferation, gene expressions of the MSCs and the growth factors content in the secretomes of the BMSCs, PDLSCs and DPSCs.

iii. To evaluate the effect of days of culture and filtration of the CM on the level of growth factors in the CM and to evaluate the proteomic profiles of all CM samples via mass spectrometry.

iv. To evaluate the effect of the collected CM on cellular metabolic activity, mineralisation potential and migration.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Cell Culture Medium and Reagents

2.1.1.1 Primary culture medium

Culture medium was prepared using alpha-modified minimum essential medium (α-MEM) (Biosera, UK) containing 1 % penicillin/streptomycin/amphotericin (100 units/mL penicillin with 100 μg/mL streptomycin and 2.5 μg/mL amphotericin) (Sigma-Aldrich, UK), 2 mM L-glutamin (Sigma-Aldrich, UK) and supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, UK).

2.1.1.2 Phosphate Buffered Saline (PBS)

PBS solution was prepared by adding 7.8g sodium chloride (Sigma-Aldrich, UK), 1.5g dipotassium phosphate (Sigma-Aldrich, UK) and 0.2g monopotassium phosphate (Sigma-Aldrich, UK) in 1 litre of reverse osmosis water. The solution was autoclaved prior to use.

2.1.2 Mesenchymal Cell Isolation

The methods for isolation of the MSCs have been reported and known to be predictable and reliable techniques (Gronthos, Mankani et al. 2000, Seo, Miura et al. 2004, Huang, Xu et al. 2015). The methods have also been used in our laboratory works and reported in previous studies (Davies, Cooper et al. 2015, Gao, Walmsley et al. 2016). The MSCs were isolated from 6-weeks-old Wistar-Han rats (Pharmaceutical Sciences Animal House, Aston University, Birmingham, UK) with averaged weight of 120grams. Dissection was performed to isolate BMSCs from the femurs, PDLSCs from the tissue (PDL) surrounding the roots of molar teeth and DPSCs from pulpal tissue of incisor teeth (Figure 2.1).

There were total of six rats dissected through out the experiments in which 2 femurs, 4 incisors and molars were extracted from each rat. The isolated MSCs were cultured until passage 6 and at each

passage, part of the cells was cryopreserved. Generally, the cells from different isolation processes were pooled together prior to the cell culture of all the experiments.

2.1.2.1 Isolation of BMSCs

The femurs were dissected by longitudinal incision of the rats' thighs and the soft tissues were removed from the bone surfaces using a scalpel blade and tweezers. Femurs were then placed in universal tubes containing sterile 10ml α-MEM. Both ends of the femur were dissected with scissors to remove the epiphyses of the bones. Primary culture medium was flushed through the long bones with a syringe (BD Bioscience, UK) with a 22-gauge needle (Appleton Woods, UK) inserted through the bones. The medium containing flushed bone marrow was collected in 15-ml tubes (Nunc, UK) and centrifuged at 900g (Eppendorf 5804R, UK) for 5 min. Supernatants were removed and the cells were re-suspended in 1 ml of primary culture medium with 20% FBS (Gao, Walmsley et al. 2016). The cell suspension was seeded in T25 flask (Nunc, UK) with addition of 4ml primary culture medium (20% FBS) and incubated in 37°C with 5% CO_2, 21% oxygen and 74% nitrogen of humidified incubator (RS Biotech). The medium was changed every 3 days until the cells became 80-90% confluent. Culture medium was changed to include 10% FBS starting passage 2 onwards. Cells were sub-cultured to next passage as describe below in section 2.1.3

2.1.2.2 Isolation of PDLSCs

All molar teeth were carefully extracted and placed in universal tubes containing 10ml α-MEM. Molar teeth were then immersed in Falcon tubes containing 5ml of trypsin/EDTA. Falcon tubes containing teeth were incubated at 37°C with 5% CO_2, 21% oxygen and 74% nitrogen of humidified incubator (RS Biotech) for 45 minutes with gentle agitation using rotary incubator (SI20H, Stuart Scientific, UK) (Gao, Walmsley et al. 2016). After 45 minutes, the enzyme action was stopped with addition of 5 ml α-MEM with 10% FBS. The solution was filtered through 70μm cell sieve (Millipore, UK) to collect the filtrate and centrifuged for 5 min with 900g. Supernatants were

removed, and the cells were suspended in 1ml of primary culture medium containing 20% FBS. The cell suspension was seeded in T25 flasks with addition of a further 4ml primary culture medium (20% FBS) and incubated at 37°C with 5% CO_2, 21% oxygen and 74% nitrogen of humidified incubator (RS Biotech). Medium was changed every 3 days until the cells became 80-90% confluent. Culture medium was changed to 10% FB from passage 2 onwards.

2.1.2.3 Isolation of DPSCs

All incisor teeth were carefully extracted and placed in a universal tube containing 10ml α-MEM. The incisors were placed on a glass slide and teeth were dissected longitudinally using a scalpel blade and the pulp was carefully extracted. The pulp tissue was minced into small pieces of ~1 mm^3 in size. Tissues were moistened dropwise with α-MEM media. The minced tissues were then placed in Falcon tube containing 4mls of 0.25% (w/v) trypsin and 1mM EDTA (Gibco, UK) and the Falcon tube was incubated at 37°C for 30 minutes with gentle agitation (Gao, Walmsley et al. 2016). After 45 min, the 0.25% (w/v) trypsin and 1mM EDTA enzymatic action was stopped by addition of 4 ml α-MEM with 10% FBS. The solution was filtered through a 70μm cell sieve to collect the filtrate and centrifuged for 5 minutes at 900 rpm. The supernatants were removed, and the cells were suspended in 1ml of primary culture medium with 20% FBS. The cell suspension was seeded in T25 flasks with the addition of a further 4ml primary culture medium (20% FBS) and incubated in 37°C with 5% CO_2, 21% oxygen and 74% nitrogen of humidified incubator (RS Biotech). The medium was changed every 3 days until the cells became 80-90% confluent. The culture medium was changed to 10% FB starting passage 2 onwards.

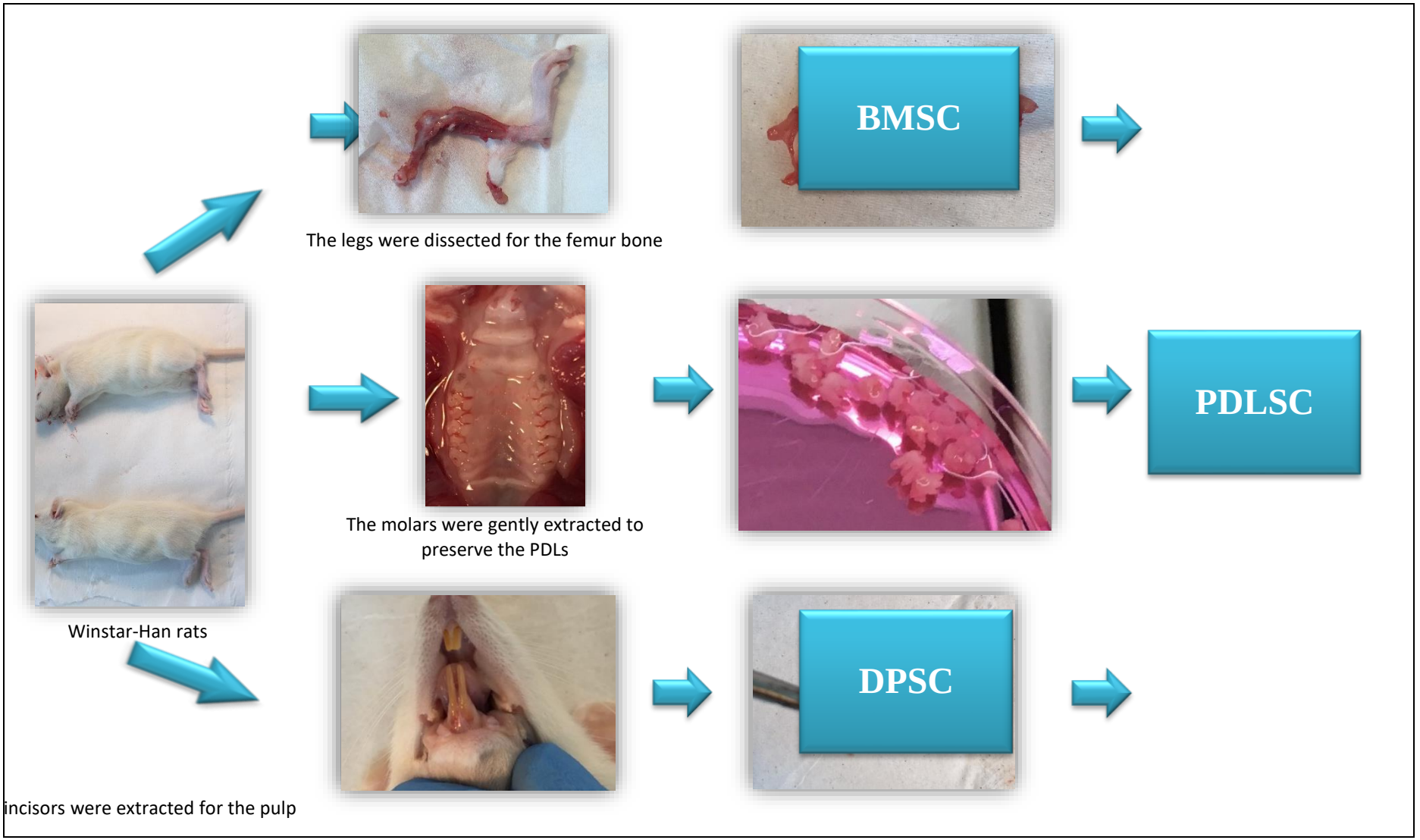


Figure 2.1 Tissue dissection for MSC isolation

2.1.3 Cell Subculture/ Passage

When the culture in T25 flasks were confluent, 1ml of 0.25% (w/v) trypsin and 1mM EDTA were added to each flask to detach the monolayer cells following removal of the culture media. Flasks were incubated for 10 min before addition of 1ml of primary culture medium to stop the trypsin/EDTA's enzymatic action. The cell suspension was centrifuged for 3 minutes at 800rpm. Supernatants were discarded and a further 3ml of primary culture medium were added to the cell pellets. The cell suspension was seeded into three T75 flasks with 1ml of cell suspension in each flask together with a further 11ml of primary culture medium. Culture medium was changed every 3 days until the cultures became 80-90% confluent.

2.1.4 Storage of Cells

Cells from each passage were cryopreserved in liquid nitrogen for future use. Prior to storage, 3ml of trypsin/EDTA (Gibco, UK) were added into the T75 flask and incubated for 10 minutes. Subsequently, 3 ml of primary culture medium was added, and the cell suspension was centrifuged for 3 minutes at 800rpm. The supernatant was discarded, and the pellet was re-suspended with 1ml of prepared medium containing 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK), 20% FBS and 70% α-MEM. The cell suspension was transferred into cryovials (Corning, UK) and stored at -80°c freezer overnight before transfer into liquid nitrogen storage (BOC Gases).

When cells were required, vials of cryopreserved cells were thawed in a 37°C water bath prior to centrifugation to obtain the cell pellet. The cell pellet was then resuspended in 1 ml of primary culture medium and seeded in T75 flasks with an additonal 15ml of primary culture medium as mentioned in section 2.1.1.1.

2.1.5 Cell Counting

Cell counting throughout the experiments was performed manually. The cultured medium in the flask was aspirated and washed with PBS three times. The cells were then trypsinized with 3ml trypsin/EDTA incubated for no more than 10 minutes. After 10 minutes, the trypsin's action was stopped with addition of 1ml of primary culture medium. The cell suspension was collected in centrifuge tube for centrifugation for 5 minutes for 800rpm. The supernatant was aspirated leaving the cell pellets at the bottom of the tube. The cell pellet was mixed with 3ml of the primary medium for cell suspension.

Then, 50 µL of a cell suspension was added to an equal volume of 0.4 % (w/v) Trypan blue cell stain in microcentrifuge tube and incubated for 10 minutes at room temperature to allow the uptake of the dye by the cells. Then, 10µl of the mixture was transferred to a haemocytometer (Improved Neubauer, Hawksley, UK). Each square of the hemocytometer represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to 1 ml, the cell concentration per ml which also implies total number of cells were determined using the following calculations.

Cells per ml = the average count per square x the dilution factor x 10^4 (count 10 squares)

The viable and non-viable cells were counted manually under a microscope (Zeiss, Germany). Five counts per sample were recorded and an average value was calculated.

2.2 Gene Expression Analysis

The cultures were used in semi-quantitative polymerase chain reaction (PCR) to determine the stem cell markers (Dominici, Le Blanc et al. 2006). The process involved ribonucleic acid (RNA) isolation and analysis steps, synthesis of the complementary DNA (cDNA), concentrating the synthesised cDNA, semi-quantitative reverse transcriptase PCR (sqRT-PCR) and relative quantification of the PCR products through gel electrophoresis analysis.

For the PCR analysis, the cells of each isolation process as in section 2.1.2 were cultured separately and process until the cDNA synthesis before the cDNA were pooled together for the gene expression analysis.

2.2.1 RNA isolation

The cells were cultured in T75 flask with 12ml primary culture medium and 10% FBS. Once the cells were 80-90% confluent, the RNA of the BMSCs, PDLSCs and DPSCs were isolated using the Qiagen RNeasy minikit (Qiagen, UK) following the manufacturer's instructions.

The media of the cell cultures were aspirated, and cultures were washed with PBS to remove the media which may disturb the lysis process. The cells were lysed with 350µl of RLT lysis buffer and the lysate was collected in the eppendorf tubes with addition of equal volume of the 70% ethanol.

The mixture of lysate-ethanol was transferred to RNeasy mini-column and centrifuged at 10,000 rpm for 30 seconds using centrifuge machine (5415D Centrifuge, Eppendorf, Germany). The excess ethanol was removed, and the cell lysate were washed with wash Buffer RW1(Qiagen, UK) and centrifuged again to eliminate the unwanted biomolecules in the samples. Then, 80µl of Deoxyribonuclease I (DNAase I) (Qiagen, UK) was added to the samples for 15 minutes as room temperature to eliminate the DNA before the samples were washed again with Buffer RW1.

The samples were washed with Buffer RPE (Qiagen, UK) and centrifuged twice to remove the traces of salts. The mini-column was placed in new collection tube and 30µl of RNase free water was added and centrifuged for 1 minutes and 10.000rpm to collect the purified RNA. The concentration and purity of the RNA of the BMSCs, PDLSCs and DPSCs were determined using Biophotometer (Eppendorf, UK).

To ensure RNA yield and integrity from the samples, the RNA produced in the above steps were visualized with gel electrophoresis procedures. An agarose gel (Web scientific, UK) were mixed

with 1x TAE buffer which is the mixture of 2M Tris-acetate and 0.05M EDTA (Qiagen, UK). The gel mixture was heated in the microwave (Sanyo, UK) for 2 minutes and cooled by placing the mixture container under running water. SYBR Gold (Invitrogen, USA) was added into the gel mixture to visualize the RNA bands. The gel mixture was poured into a tape-dammed tray with comb to generate wells and allowed to set at room temperature.

RNA mixture was prepared by adding 1 µl of RNA with 3µl of RNase water and 1µl of 10x loading buffer ((Promega, UK) and the mixture was loaded into the wells in the agarose gel. The gel was placed in the electrophoresis tank and eletrophoresed for 30 minutes at 120V before transferred to G:Box (Syngene, USA) for visualisation of the RNA bands.

2.2.2 Reverse transcription PCR for sysnthesis of the cDNA

The RNA samples were then used for cDNA synthesis via reverse transcription process of the PCR. This process was performed using the Tetro kit following the manufacturer's instructions (Bioline, UK). The reverse transcription mastermix was prepared by adding 1µl of Oligo $(dT)_{18}$ primer, 1µl of 10mM deoxynucleoside triphosphates (dNTP), 4µl 5x Reverse Transcriptase Buffer, 1µl RNase inhibitor and 1µl of Reverse Transcriptase (200u/µl). 2µg of RNA was used per sample and added to RNase free water to produced final volume of 12µl. The 8µl of the mastermix was added to the 12µl prepared RNA sample and incubated in thermal cycler (Mastercycler, Eppendorf, Germany) at 45°C for 30 minutes for annealing of the cDNA followed by 85°C for 5 minutes for activation of the DNA polymerase. The activation of DNA polymerase allows extension of the DNA template by adding complementary dNTPS at 5' to 3' direction. The samples cooled to 4°C before used in next PCR steps or stored in -80°C.

2.2.3 Concentrating the cDNA

The cDNA was added with 480µl of RNase free water to achieve total volume of 500µl before placed in 0.5ml microcon filter column (Amicon Ultra, Milipore, USA) for centrifugation. The sample was centrifuge for 2 minutes at 10 000 rpm, then 1 minute at 8 000 rpm to eliminate the excess water until the final volume of 50 to 60 µl of cDNA sample. The filter coulumn was inverted in new clean microtube and centrifuged at 800rpm for 1 minute to collect the concentrated cDNA. The cDNA was stored at -20°C for future use.

2.2.4 Semi-quantitative reverse transcriptase PCR

Initial step of the reverse transcriptase PCR involved preparation of the PCR master mix. The RedTaq mastermix (Sigma Aldrich, UK) was prepared with additional of the primers as in Table 2-1.

Reagent	Volume
REDTaq (Sigma Aldrich,UK)	12.5µl
1µM Forward Primer (Invitrogen, UK)	1µl
1µM Reverse Primer (Invitrogen, UK)	1µl
RNase free water	9.5µl

Table 2-1 The PCR REDTaq mastermix preparation

The mastermix was aliquoted to 6µl of volume in each 0.2ml PCR tubes (Appleton Woods, UK) with additional of 50-100ng of the cDNA. The mixture was mixed thoroughly with vortex mixer and transferred to Mastercycler Gradient Thermal Cycler for gene amplification. The details of the thermal cycles are listed in Table 2-2 and details of the primers used for gene analysis in this PhD study is lited in Table 2-3.

T°C	Duration	Activities
95°C	5 minutes	Initial denaturation of the cDNA
94°C	20 seconds	Separation of the double stranded DNA (dsDNA) to single stranded DNA (ssDNA)
60.5°C	20 seconds	Annealing of the selected primers to ssDNA
68°C	20 seconds	Primer extension by Taq polymerase enzyme
The cycles repeated for 21-35 cycles depending on the primers before entering the final cycle as below:		
72°C	10 minutes	Extension phase

Table 2-2 The protocol steps in RT PCR

Primer	Sequences (5' to 3')	Annealing T (°C)	Number of cycles	Reference
Housekeeping Gene				
GAPDH	F-CCCATCACCATCTTCCAGGAGC R-CCAGTGAGCTTCCCGTTCAGC	60.5	27	(Davies OG 2014)
Stem Cell Markers				
CD105	F-TTCAGCTTTCTCCTCCGTGT R-TGTGGTTGGTACTGCTGCTC	60.5	39	(Davies OG 2014)
CD90	F-AGCTCTTTGATCTGCCGTGT R-CTGCAGGCAATCCAATTTTT	60.5	27	
CD29	F-AATGGAGTGAATGGGACAGG R-TCTGTGAAGCCCAGAGGTTT	60.5	27	
c-myc	F-CTTACTGAGGAAACGGCGAG R-GCCCTATGTACACCGGAAGA	60.5	36	
CD44	F-TGGGTTTACCCAGCTGAATC R-CTTGCGAAAGCATCAACAAA	60.5	36	
Growth Factor Genes				
VEGF	F- TTCGTCCAACTTCTGGGCTC R- GCAGCCTGGGACCACTTG	60.5	39	(http://biotools.umassmed.edu/bioapps/primer3_www.cgi)
TGF-β1	F- CGCCTTAGCGCCCACTGCTCCTGT R-GGGGCGGGACCTCAGCTGCAC	60.5	33	
IGF-1	F- GACCCGGGACGTACCAAAAT R- GTACTTCCTTTCCTTCTCCTTTGC	60.5	36	
IGF-BP1	F- ACCTCAAGAAATGGAAGGAGCC R- ACACAGACCTGTGGGATTCG	60.5	42	
Osteogenic Genes				
ALP	F- CTCCGGATCCTGACAAAGAA R- ACGTGGGGGATGTAGTTCTG	60.5	30	(Davies OG 2014)

Osteonectin	F-AAACATGGCAAGGTGTGTGA R-AGGTGACCAGGACGTTTTTG	60.5	24	
Core binding factor alpha 1(cbfα1)	F-GCCGGGAATGATGAGAACTA R-GGACCGTCCACTGTCACTTT	60.5	36	
Hypoxic Marker				
HIF-1α	F-AGCGATGACACGGAAACTGA R-ATCAGTGGTGGCAGTTGCG	60.5	27	(http://biotools.umassmed.edu/bioapps/primer3_www.cgi)

Table 2-3 List of primer sequences used in sqRT-PCR analysis

2.2.5 Relative quantification of the PCR products

The PCR products were transferred to the 1.5% agarose gel which was prepared by adding 0.9g agarose with 60ml of 1x TAE buffer with 0.5µg/ml ethidium bromide as in section 2.2.1. The gel was electrophoresed for 30 minutes at 120V. The gel was transferred into the G:BOX for visualisation by UV illumination and the image of the PCR product's bands were captured with Genesnap software (syngene, USA). The analysis of the band intensity was performed using GeneTools software (Syngene, USA) and exported to Excel (Microsoft, USA). The normalisation of the specific gene's band intensity was normalised to the band intensity of the GAPDH.

2.3 Multi-lineage Differentiation of MSCs

MSCs should be able to differentiate into osteogenic and adipogenic cell-lineages. For the differentiation experiments, the MSCs were seeded into 35mm petri dishes at a density of $2.5x10^5$. Once the cells reached 80% confluency, the cells were treated with induction media as listed in Table 2-4 after 3 times washing with PBS. The MSCs in passage 3 were used in this experiment.

The supplements in the media were filtered with 0.2µm filter membrane for sterilization. Cells were induced for differentiation in the incubator (RS Biotech) and the media was changed every 3 days for up to 21 days.

	Ingredients
Osteogenic Differentiation Media	α-MEM with 10% FBS 1 % penicillin/streptomycin/amphotericin 50 µg/ml ascorbic acid (Sigma-Aldrich, UK). 10^{-8}M dexamethasone (Sigma-Aldrich, UK) 10 mM β-glycerophosphate (Sigma-Aldrich, UK).
Adipogenic Differentiation Media	α-MEM with 10% FBS 1 % penicillin/streptomycin/amphotericin 0.5 mM 1-methyl-3-isobutylxantine (Sigma-Aldrich, UK) 60 µM indomethacin (Sigma-Aldrich, UK) 0.5µM hydrocortisone (Sigma-Aldrich, UK)
Control Media	α-MEM with 10% FBS 1 % penicillin/streptomycin/amphotericin

Table 2-4 List of ingredients in differentiation and control media

2.3.1 Alizarin Red Staining

After 3 weeks of osteogenic induction, the differentiated cells were fixed with 10% neutral buffered formaldehyde (Leica Biosystems, UK) for 15 minutes prior to staining procedure. The stain solution was prepared by adding 2 mg/mL of 40mM ARS with 0.5 M acetic acid (BDH Laboratory Supplies, UK). The pH of the solution is adjusted to 4.2 using 1 % (v/v) ammonium hydroxide (BDH Laboratory Supplies, UK). The cultures were washed twice with PBS and stained with alizarin red S (ARS) for 20 minutes before washed with distilled water for 4 times. The procedures were performed under room temperature. The stained calcified nodules were examined under a phase-

contrast microscope (Axiovert 25, Zeiss, UK) where the images were taken at least triplicate in each well and the area with most abundance of calcified nodules were presented in result section.

2.3.2 Oil Red O Staining

Similar procedures were applied after cells were induced for adipogenic. Oil Red O (ORO stain) were used to visualize lipid droplets accumulated in adipocyte-like cells formed after adipogenic differentiation. The ORO stock solution was prepared by mixing 0.5mg ORO stain (VWR, UK) with 200mL isopropanol (VWR, UK). The solution was heated up for 1 hour at 56℃ and later was cooled down to room temperature. Three parts of the stock solution was added to 2 parts of distilled water as working solution and filtered to remove any undissolved dye.

Prior to the staining procedure, the differentiated cells were fixed with 8% formalin for 10 minutes in 4°C. The fixed cells were washed with PBS solution 3 times before addition of the stain solution for duration of 40 minutes at room temperature with gentle agitation. Removal of the ORO dye was performed by washing the stained cells with 60% isopropanol (Sigma-Aldrich, UK) and followed by PBS solution. The cultures were then placed under the phase-contrast microscope (Axiovert 25, Zeiss, UK) and the presence of stained lipid droplets were observed.

2.4 Cell Culture in Hypoxia

MSC cultures were used after reaching 80-90% confluency. The cells in 75mm^2 were trypsinized for 10 mins with 3ml of trypsin/EDTA until the cells detached from the flask surfaces. Then, 3ml of primary culture medium were added into the flask to stop the trypsin's action. The cell suspension was collected and centrifuged for 4 mins at 800rpm. The supernatants were removed, and the cell pellets were suspended with a further 5ml of primary culture medium. The cell suspension (10µl) was added to an equal volume of 0.4 % (w/v) Trypan blue cell stain (Sigma-Aldrich, UK). The suspension was mixed thoroughly using a Gilson pipette. Then the suspension was transferred to a Neubauer haemocytometer (Hawksley, UK) and the viable cells were counted under a variable relief contrast (VAREL) microscope (Zeiss Axiovert 25) (Zeiss, UK). Five counts per sample were recorded and an average calculated. Suspensions were then diluted according to the required seeding density.

The cells were then seeded into 35mm dishes with cell density of 2.5 x 10^4 cells/ml. On day 0, the primary culture medium (with 10% FBS) was used as mentioned in section 1.1.1 to help the attachment of the cells in each well and half of the prepared wells were placed in normal incubation with 37°C with 5% CO_2, 21% oxygen and 74% nitrogen of humidified incubator (RS Biotech) and another half were incubated in the hypoxia incubator with 37 °C in 5 % CO_2, 2% oxygen and 93% nitrogen incubator (Galaxy 48R, New Brunswick). Passage 3-5 of BMSCs, DPSCs and PDLSCs were used in these experiments which were performed in triplicate.

The culture media in each well was refreshed every 3 days. Three wells of cell culture from each incubation were trypzinized and cell counts as in section 2.1.5 were performed to monitor cell growth. The cell counting was repeated every three days until the twelfth day.

2.5 Cell Culture in Different Serum and Oxygen Concentrations

The BMSCs, PDLSCs and DPSCs were used in another experiment to evaluate the cell growth and growth factor production in different serum concentration and different oxygen incubation. The cells

were seeded into 35mm dishes with cell density of 2.5 x 10^4cells/ml with 10% FBS primary culture media. The cultures were incubated either in in normal incubation with 37°C with 5% CO_2, 21% oxygen and 74% nitrogen of humidified incubator (RS Biotech) or in the hypoxia incubator with 37 °C in 5 % CO_2, 2% oxygen and 93% nitrogen incubator (Galaxy 48R, New Brunswick).

After one day, primary culture medium was prepared with two different FBS concentration which were 10% and 0.5%. The culture medium in each well was discarded and the cultures were washed with 1 ml PBS to ensure total removal of the old media. Then the media were changed with 2 ml of the prepared culture medium. Three wells were used in each different serum concentration from both incubations.

On Day 3, the conditioned media (CM) were collected and centrifuged for 5 minutes at 1500rpm and stored in -20°C until analysed for growth factors levels with ELISA. The number of viable cells of each well were counted and recorded as described above.

Passage 3-5 of BMSCs, DPSCs and PDLSCs were used in these experiments which were performed in triplicate.

2.6 Cell Culture in Serum Free Media

2.6.1 Proliferation of the Cells in Serum Free Media

Passage 4 of BMSCs, PDLSCs and DPSCs were used in these experiments which were performed in triplicate. Cells were seeded in 12-well plate with cell density of 1.0 x 10^4 per ml with 800µl of 10% FBS primary culture medium and incubated in normal incubation with 37°C with 5% CO_2, 21% oxygen and 74% nitrogen of humidified incubator (RS Biotech). After one day, the media of the cultures were removed and washed with PBS three times before 800µl of serum free media was added in each well. The MSCs were incubated for 24, 48 and 72 hours in normoxia (21% oxygen)

and hypoxia (2% oxygen) incubations before the proliferation of the MSCs in serum free media were evaluated by doing cell count as described in section 2.1.5.

2.6.2 Cell culture for CM Collection and Gene Expression Analysis

For CM collection, passage 4 of BMSCs, PDLSCs and DPSCs were seeded in 12-well plate with cell density of 1.0 x 10^4 per ml with 800µl of 10% FBS primary culture medium and incubated in normal incubation with 37°C with 5% CO_2, 21% oxygen and 74% nitrogen of humidified incubator (RS Biotech).

Once the cells have reached 80% confluent, the media were changed with serum free media. Prior to that, the dishes were washed three times with 400µl of PBS. The CM was collected from four dishes after either two days of three days of incubation. The collected CM were centrifuged for 1500rpm for 5 min and stored at -20°C for later growth factor analysis. As one of the objectives of the study was to evaluate the effect of the filtration to the growth factor levels in the CM, part of the collected CM was also filtered with 0.2µm membrane before kept in -20°C until analysed for ELISA.

The cells were used for RNA isolation and semi-quantitative PCR as mentioned in section 2.2 for hypoxic marker and growth factors genes expression.

2.7 Collection of the Cultured Media (CM) under Various Conditions

The conditioned media (CM) which contain the secretomes were collected for further protein analysis and functional assays. In general, the collection was performed after the cells (BMSCs, PDLSCs and DPSCSs) were cultured with 10% serum media in T75 flask until 70-80% confluent. The cells were washed three times with 3ml of PBS before 15 ml of serum serum media were added into each flask. The cells were incubated in either normal incubator with 21% oxygen, or in the hypoxic incubator in which the oxygen concentration was set to 2%. In order to evaluate the duration

of incubation on the secretome, the CM were collected from both normal and hypoxic incubators on second and third day of culture.

The CM were then collected in every flask and transferred into separate 50ml tubes based on the incubation types (Normoxia Vs Hypoxia), cell-types (BMSCs, PDLSCs and DPSCs) and different collection days (Day 2 CM Vs Day 3 CM) before kept in -20°C until used.

2.8 ELISA of Growth Factors

The CM were analysed for their VEGF, TGF-B1 and IGF-1 content using commercially available rat ELISA kits (R&D Systems). The steps of the ELISA procedures were done according to the protocol provided with the kits.

In brief, the collected CM were thawed at room temperature. The CM and prepared rat VEGF standards were pipetted into the 96-well plate provided by the manufacturer. The plates have been pre-coated with monoclonal antibody specific for rat VEGF and blocked with blocking buffer prior to addition of the secretomes. After incubated at room temperature on the horizontal orbital microplate shaker for 2 hours, the plates were washed 5 times with autowasher. Then polyclonal antibody conjugated to horseradish peroxidase were added into each well and incubated for another 1 hour before the plates were washed again and added with substrate solution. The colour of the solutions in each well turned into blue with these enzymatic reactions between the polyclonal antibody and the substrates. After 30 minutes, the enzymatic reactions were stopped with additional of diluted hydrochloric acids, which turned the blue solutions into yellow. Then, the optical density (OD) of each well were determined by microplate reader at 450nm wavelength.

Based on the OD and VEGF concentrations of the standards, a standard curve graphs were plotted. From the formula given on the graph generated with Microsoft Excel, the VEGF concentrations of each sample were calculated and averaged.

The ELISA procedure for TGF-β1 was almost similar as the one reported for VEGF but with additional step prior to the start of the ELISA protocol. The TGF-B1 in each sample was activated with 1 N Hydrochloric acid for 10 minutes before neutralized with 1.2 N Sodium hydroxide/0.5 M HEPES. This step was important because the latent TGF-B1 in the secretomes must be activated to immunoreactive TGF-B1. TGF-B1 that was immunoreactive could be detected by the immunoassay. Other than reactivation of the latent TGF-B1, the other different between TGF-B1 protocol than VEGF's was the incubation time for conjugated polyclonal antibody took two hours rather than one hour as in VEGF's protocols.

The basic ELISA procedure for IGF-1 was also similar as VEGF and TGF-β1, except that the samples were diluted 2-fold with the diluent provided by the manufacturers. The incubation time for conjugated polyclonal antibody was also two hours. The concentration IGF-1 that was calculated via the OD of the solutions and the generated standard curve similar as the one generated for the VEGF and TGF-β1 except that the value was multiplied with the dilution factor which was two.

2.9 Protein quantification of the collected CM

The quantity of the total proteins in the collected CM was measured using colorimetric quantification of the bicinchoninic acid or BCA Assay (Thermo Scientific, UK) following the protocol suggested by the manufacturer. Prior to the assay, the bovine serum albumin (BSA) was prepared into several protein concentrations to act as the standard. The prepared concentration of the BSA standards by mixing 2mg/ml of the BSA stock solution with the diluent according to the volume suggested by the manufacturers.

Then, the working reagent (WR) of the BCA was prepared by adding 20 parts of Reagent A (that contains the BCA, sodium carbonate, sodium bicarbonate and sodium tartrate in 0.1M sodium hydroxide) with 1 part of the Reagent B that contains 4% cupric sulfate. The collected CM (samples) as well as the prepared standards were then pipetted for 10µl into each well of 96-well plate in

triplicate before 80µl of the WR were added. The solutions in each well were mix gently on the plate shaker for 30 seconds. The plate was incubated in 37°C for 30 minutes and the solutions in each well changed to purple colour depending on the protein content in the samples or standard solutions. The colour changes in the solution was due to reduction of the Cu^{2+} ions from the cupric sulfate to Cu^{+} ions, which later bind to the two molecules of BCA and formed the purple coloured solutions. The absorbance of the solution was measured at 570nm wavelength using the spectrophotometer machine (Biotek, UK).

Based on the absorbance reading of the standards, standard curve was plotted using Microsoft Excel and the formula was generated. The protein concentration in each sample was calculated using the formula.

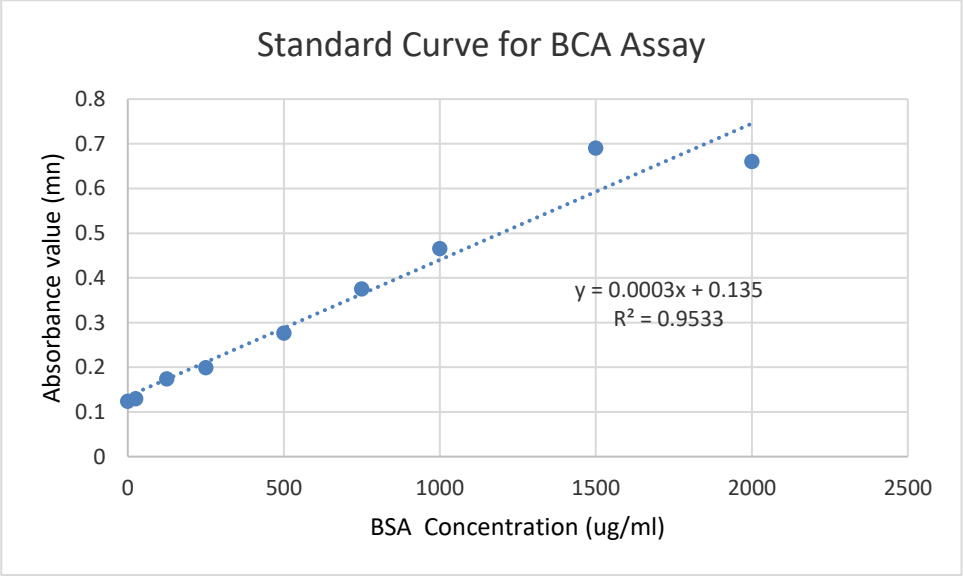


Figure 2.2 Example of the standard curve for the BCA assay to generate the formula for protein sample calculations.

2.10 Mass spectrometry of the collected CM

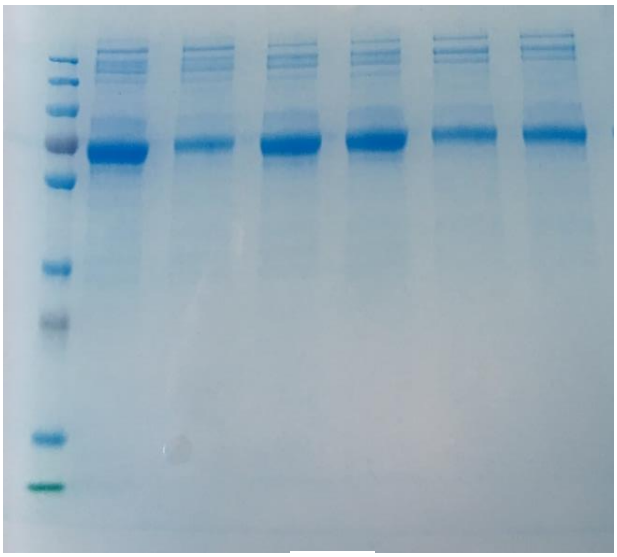
The collected CM from Day 3 and normoxia incubation (BMSCs, PDLSCs and DPSCs) were sent for proteomic analysis through liquid chromatography mass spectrometry (LC-MS/MS) in the Advanced Mass Spectromtery Facility, University of Birmingham. Prior to sending the sample for mass spectrometry, the collected CM were concentrated 10x using the Amicon® Ultra Filter 3kDa (Merck Milipore, UK) and centrifuged for 30 minutes at 14 000 g.

The protein level of the concentrated CM were measured as in section 2.9 and 10µg/ml protein of the concentrated CM was mixed with the Laemmli Buffer (Sigma-Aldrich, UK) in the ratio of 1:1 and the mixture was heated for 5 minutes in water bath at temperature 95°C.

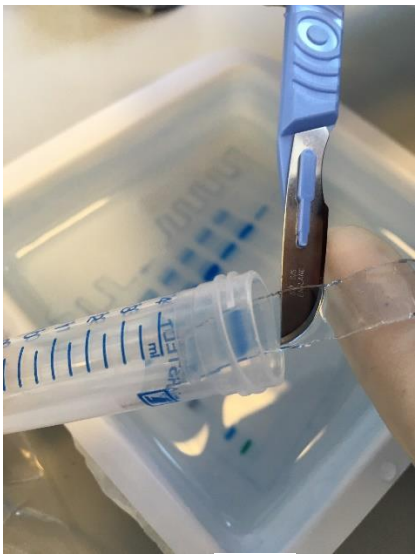
Then, 20µl of the sample was transferred into gel electrophoresis for 30 minutes with 200V. Upon completion of the electrophoresis process, Mini-Protean TGX precast gels from Bio-Rad, Hertfordshire, United Kingdom with gel composition of 12% acrylamide were used (Bio-Rad, UK) was soaked in the InstantBlue™ Protein Stain (Expedeon, UK) for 1 hour until the protein bands were visible on the gel as in Figure 2.3(a). The gel was then washed three times with distilled water to remove excessive dye before each sample column of protein bands was cut and placed in the 15ml Falcon tubes Figure 2.3(b). The samples were sent to the Advanced Mass Spectromtery Facility, University of Birmingham for further sample preparation that involved protein digestion and desalting that produced peptides. The peptides were loaded into the liquid chromatography column attached to the Q Exactive HF mass spectrometry machine (ThermoFisher Scientific, Paisley, United Kingdom) as in Figure 2.3(c). The mass spectrometry process was performed by the mass spectrometry officer (Dr Jinglei Yu) in technical duplicates (Appendix 1).

The principle of the mass spectrometry is based on the measurement of the peptides based on its mass (m) over charge (z) ratio and detected by the detector and produced a spectrum of of the peptide molecules and its fragments. The mass spectrum was then searched against the protein database for the list of proteins detected in the sample.

The mass spectrum in this experiment was searched against the Sequest and Mascot for rat protein database (European Bioinformatics Institute, Cambridge, United Kingdom). The search results from each of the technical replicates were combined and proteins which were identified with two or more unique peptides were classed as identified. Only unique peptides were used for protein quantification (performed in Proteome Discoverer) and protein grouping was employed (only proteins which contained unique peptides were used). The final list of protein was corrected with the protein listed in negative control sample.



a)



b)

c)

Figure 2.3 The images of the gel loaded with samples before sent to mass spectrometry (a) The protein bands of each sample upon completion of the electrophoresis. (b) The gel was cut to six colums based on the protein bands and put it in the 15ml Falcon tube. (c) The mass spectrometry machine in the Advanced Mass Spectromtery Facility, University of Birmingham, UK that was used for the proteomic analysis.

2.11 Tandem Mass Tag (TMT) Mass Spectrometry

Based on the data that we received from mass spectrometry of the 3 samples in section 2.10, we extended our method to the quantitative mass spectrometry analysis. We used Tandem Mass Tag™ (TMT) 10 plex kit (Thermo Scientific, UK) to label the peptides from every sample before the mass spectrometry process begun. The TMT reagent set contains ten different chemical compounds that have same mass and chemical structure but different combinations of ^{13}C and ^{15}N isotopes in mass reporter arm. The output of the TMT Mass Spectrometry enables the relative quantitation of each sample protein in comparison to the control sample. The control sample for this experiment was a pool made of a small number of samples.

Before the ten samples were labelled with the TMT, the protein samples were digested. The digestion process started by adding 100µg of each sample into eppendorf tube with 100mM of triethyl ammonium bicarbonate (TEAB) (Sigma -Aldrich, UK) and made up to a final volume of 100µl. Then, 5µl of 200mM tris(2-carboxylethyl) phosphine (TCEP) (Sigma -Aldrich, UK) was added into each sample to reduce any disulphide bonds. The samples were vortexed before incubating at 55°C for 1 hour. Then 5µl of 375mM iodoacetamide was added to the samples and incubated for 30 minutes in the dark and at room temperature, to alkylate the reduced cysteines. 5µl of 230mM of dithiothreitol (DTT) was then added into the solution samples to quench any unused iodoacetamide and incubated again for 10-15 minutes at room temperature. Finally, the samples were digested by adding the trypsin at the ratio of 1:40 with the samples and incubated overnight at 37°C.

TMT reagents were prepared with anhydrous acetonitrile as recommended by the manufacturer. Each sample was labelled with a separate TMT label for 1 hour at room temperature. Following this 5% hydroxylamine was added to quench the reaction between the TMT reagents and samples. The labelled peptide samples were then pooled together at equal volume and dried in a vacuum centrifuge. Then, the sample was cleaned up using desalting column and sent to the Advanced Mass

Spectromtery Facility, University of Birmingham. The peptides were loaded into the liquid chromatography column attached to the Q Exactive HF mass spectrometry machine (ThermoFisher Scientific, Paisley, United Kingdom).

The output of the mass spectrometry was searched using Proteome Discoverer (version 1.4 and 2.1 ThermoFisher Scientific, Paisley, United Kingdom) against the rat protein database for each technical replicate and the search results were combined. Relative protein quantification was perfomed using Proteome Discoverer Software based on the abundance value of each protein that was normalised to the abundance value of the control sample.

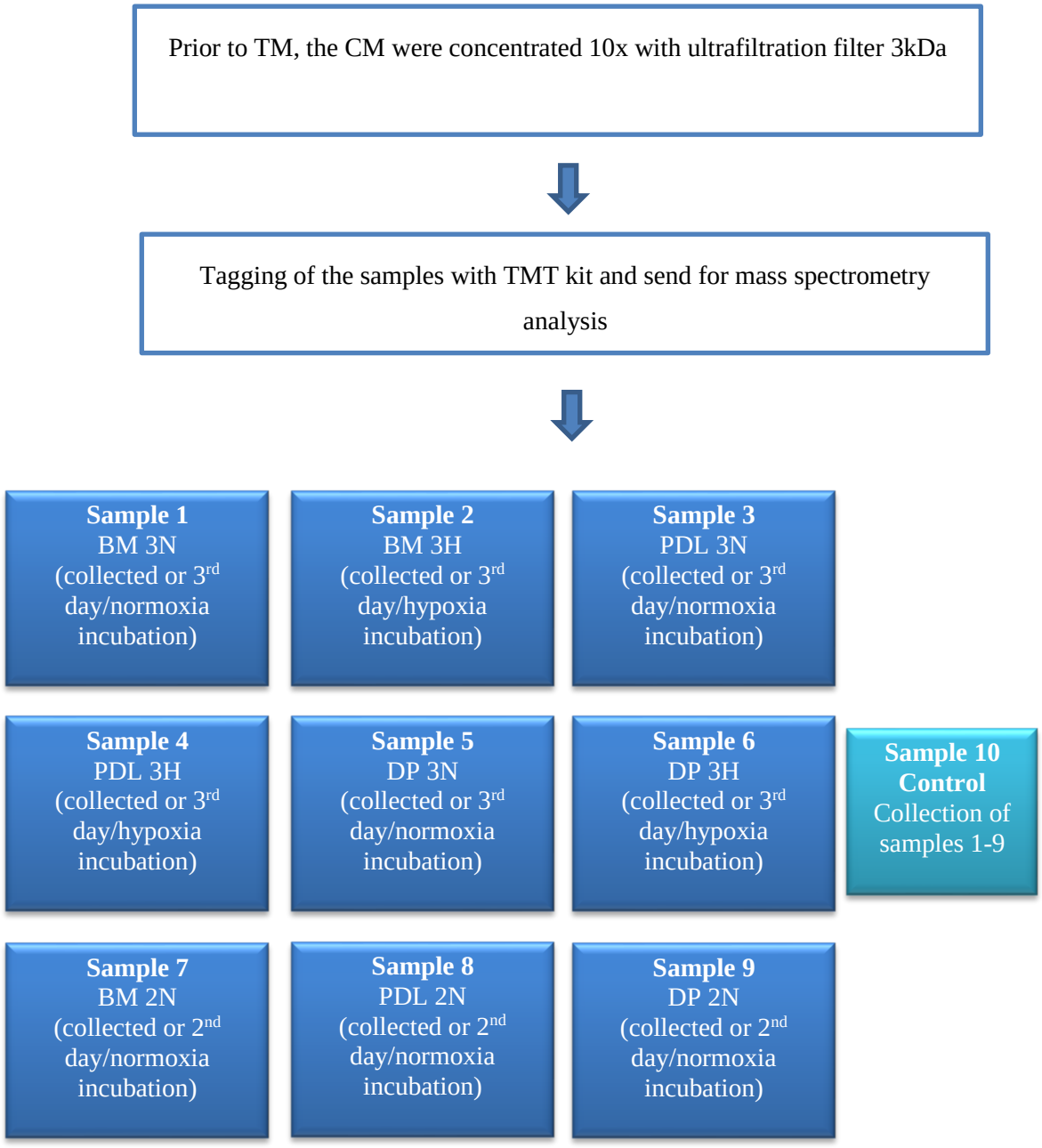


Figure 2.4 The schematic diagram of the TMT Mass Spectrometry process

2.12 Analysis of Bioactivity of CM using the MTT Assay

Pre-osteoblasts cells (MC3T3) were seeded in three 96-well plates with 10,000 cells per well in 100µl α-MEM (10% FBS) and incubated in normal incubation with 37°C with 5% CO_2, 21% oxygen and 74% nitrogen of humidified incubator (RS Biotech). After 24 hours, the media in each plate was removed and the wells were washed three times with 100µl PBS to ensure complete removal of serum media.

Prior to that, protein content in each collected CM were calculated using BCA assay as mentioned in section 2.9 before 100µg/ml of proteins CM were added into microcentrifuge tubes with SFM. The mixture was vortexed for 10 seconds before added 100µl into each well. There were 6 replicates for each CM (Normoxia and Hypoxia). The plates were incubated in normal oxygen incubation for three days and each plate was used for MTT assay to evaluate the cells metabolic activity once cultured with the nomoxic and hypoxic collected CM.

The MTT assay used a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or Thiazol Blue Tetrazolium Bromide (MTT) which produced formazan product once reacted with a NADPH enzyme produced by the mitochondria of the cells. The formazan is an insoluble purple product which is measurable by spectrophotometer machine as an indicator for cells metabolic activity. The MTT solution (5 mg/mL) was prepared by mixing the MTT powder with PBS solution and sterile filtered through a 0.2 µM filter. The solution was kept in the dark at 4°C before used.

After 1 day with serum free media, 15µl of the MTT solution was added to the cell cultures and left for incubation for another 4 hours before 50µl of DMSO was added into each well and the absorbance was determined using spectrophotometer (Biotek, UK) with the wavelength of 570nm. The MTT assay were repeated on second and third day.

Other than 100µg/ml of proteins CM, 200µg/ml of the protein CM were also tested in the MTT assay in order to evaluate the effect of different protein concentration in CM to the cell metabolic activity.

2.13 Analysis of Bioactivity of Collected CM using the ALP Assay

Alkaline phosphatase (ALP) activities of the pre-osteoblast MC3T3 cells cultured in the concentrated CM were evaluated using the SIGMA*FAST*™ p-Nitrophenyl phosphate tablets (p-NPP) (Sigma-Aldrich, UK).

The ALP assay is based on the interaction between the enzyme ALP from the cells with the substrate p-NPP from the kit. Prior to the assay, the substrate solution containing 1mg/ml of p-NPP was prepared by mixing the p-NPP tablet with 0.2M Tris buffer (provided in the kit) with 5ml of distilled water. The solution was vortexed for 5 minutes for thorough mixing.

Pre-osteoblasts cells (MC3T3) were seeded in 96-well plate with 10,000 cells per well in 150ul α-MEM (10% FBS). After 24 hours, the media in each plate was removed and the wells were washed three times with 100ul PBS to ensure complete removal of serum media. Then, 100ug/ml proteins of collected CM which were mixed SFM were added in the wells (5 wells per CM). The osteogenic media were used as control positive media and SFM were used as negative control. The cells were incubated for another three days before the cells were lysed with 0.1% Triton x-100 and incubated with prepared p-nitrophenyl phosphate solution (Sigma-Aldsrich, UK) for 1 hour in 37°C. The result of the reaction is p-nitrophenol production, and it was quantified with automatic plate reader at the 405nm wavelength with prepared standards of p-nitrophenol.

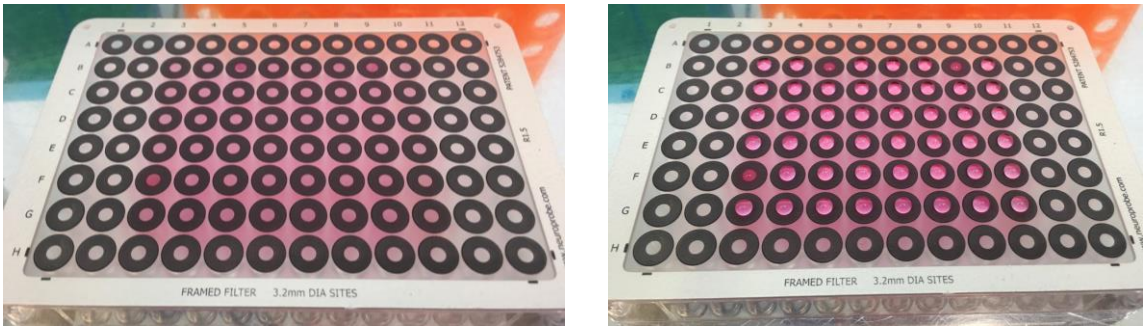
2.14 Analysis of Cell Migration bioactivity of CM using

2.14.1 Transmembrane migration assay

In order to evaluate the effect of the CM to the migration rate of MC3T3, transwell migration assay was performed (Smith 2012). The collected CM (100ug/ml) were mixed with SFM and 150µl of the mixture were added into the wells of a 96-well micro-chemotaxis plate with 8µm-pore size (Neuro Probe, UK). The MC3T3 cells were seeded with cell density of 3 x 10^4 cells/ml

in the upper chamber on the membrane (Neuro Probe, UK) with 25µl of serum free media. The plate was incubated at 37°C with 5% CO_2, 21% oxygen and 74% nitrogen of humidified incubator for 24 hours to allow migration to occur. For each CM of different cell lines (BMSCs, PDLSCs and DPSCs) and different incubation type (Normoxia/Hypoxia), six replicates were used.

After 24 hours, cells on the surface of the membrane were removed together with the membrane. The Calcein AM (1mg/ml) (Biotium Inc, UK) was added into each well at final dilution ratio 1:500 for 30 minutes. This was to label the migrated cells before luminescence measurement were performed with a Twinkle LB970 Luminometer (Berthold Technologies, UK) at absorbance wave of 485/535. The experiments were repeated twice.



a) b)

Figure 2.5 The Nuroprobe micro chemotaxis plate
a)The plate before placement of the cells on the membrane, b)The plate after placement of the cells on the membrane

2.14.2 Migration Assay with IBIDI® Inserts

Another migration assay was performed to evaluate the effect of the collected CM. The MC3T3 cells were seeded with cell density of 1 x 10^5 cells/ml in 24-well plates and the IBIDI® inserts placed in the middle of the well (Figure 2.6a). The media used was primary culture media with 10% FBS and the cultures were incubated for 24 hours at 37°C with 5% CO_2, 21% oxygen and 74% nitrogen of humidified incubator.

The media was later changed to 100ug/ml proteins of collected CM plus SFM after thorough washing with PBS. The media for control positive and negative groups were 10% FBS primary culture media and SFM respectively. The inserts were also removed, and the image of the gap created by the inserts between cells confluent mono-layer at the middle of each well as in Figure 2.6(b) were photographed with phase-contrast microscope (Axiovert 25, Zeiss, UK) and measured using Image J software as the baseline data. Then, the cultures were incubated again for another 24 hours before the gap between cells in each group was re-measured.

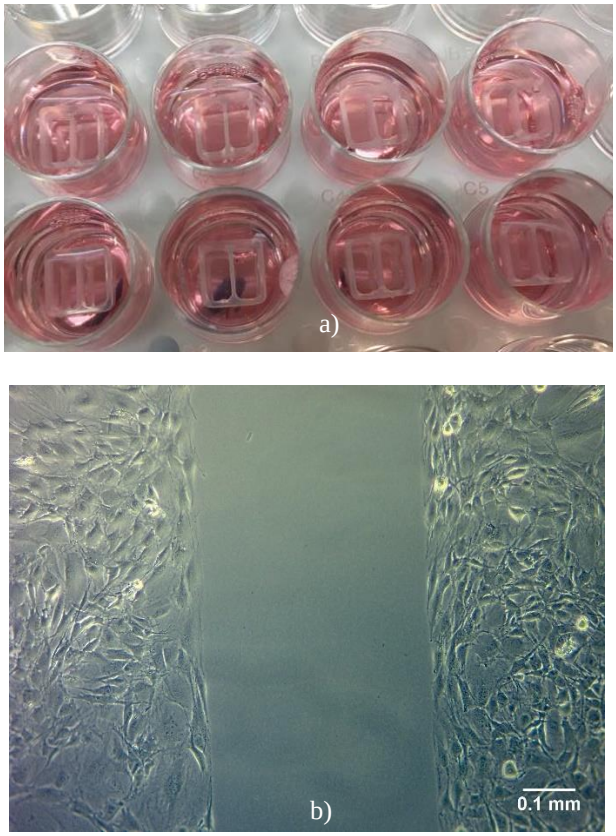


Figure 2.6 The IBIDI® inserts in the 24 well plate before migration assay started (a) and the gap created by the IBIDI® inserts between cell monolayers (b).

2.15 Analysis of Data

Data were analysed with SPSS Version 22 for Windows using independence sample t-test for analysis involving two groups and One-way ANOVA for analysis of groups more than 2. Post-hoc test (Bonferoni) was performed whenever ANOVA test was used. A p-value of <0.05 was used to indicate statistical significance.

CHAPTER 3

RESULT 1

Characteristics of mesenchymal stem cells from bone marrow, periodontal ligament and dental pulp

3 RESULTS I

3.1 Introduction

Secretome of MSCs have been studied in the last ten years and most of the studies used secretome from conditioned media of BMSCs (Chen, Tredget et al. 2008, Timmers, Lim et al. 2011). The secretome study on DPSCs were also emerging (Paschalidis, Bakopoulou et al. 2014, Fujio, Xing et al. 2015, Ahmed, Murakami et al. 2016) but there were few studies using PDLSCs (Rajan, Giacoppo et al. 2016, Ratajczak, Hilkens et al. 2016, Xia, Tang et al. 2016). There is no study that evaluates the secretomes from BMSCs, PDLSCs and DPSCs. Prior to evaluation of the secretome from these three sources, the initial characteristisation of the DPSCs and PDLSCs were compared with the BMSCs as BMSCs is the one of the well-studied stem cells.

We used rat primary cells because analysis of the cells from different tissues are best obtained from the same individual of any species. This could reduce inter-individual variabilities for comparison of the cell characteristics, cell growth and gene expression of these three cells.

The objective for this chapter was to evaluate the characteristics of BMSCs, PDLSCs and DPSCs in terms of the gene expression for stem cell markers, growth factors and osteogenic markers. In every aspect of this result chapter, BMSCs were used as the control reference cells.

The BMSCs, PDLSCs and DPSCs of passage 3 were cultured and the cells were used for PCR analysis as in section 2.2 for analysis of the stem cell marker, osteogenic and growth factor genes.

The MSCs of passage 2 were also seeded until passage 3 before tthe MSCs were seeded into 35mm petri dishes at a density of $2.5x10^5$. Once the cells reached 80% confluency, the cells were treated with induction media for osteogenic and adipogenic differentiation as detailed out in section 2.3. The experiments were repeated three times. The im

3.2 Primary Cell Culture

The phase-contrast microscope images show the appearance of all three cell-lines during passage 3 and all three cell-lines were heterogeneous in morphology, in which majority of the cells are irregular and looked like spindles (Figure 3.1).

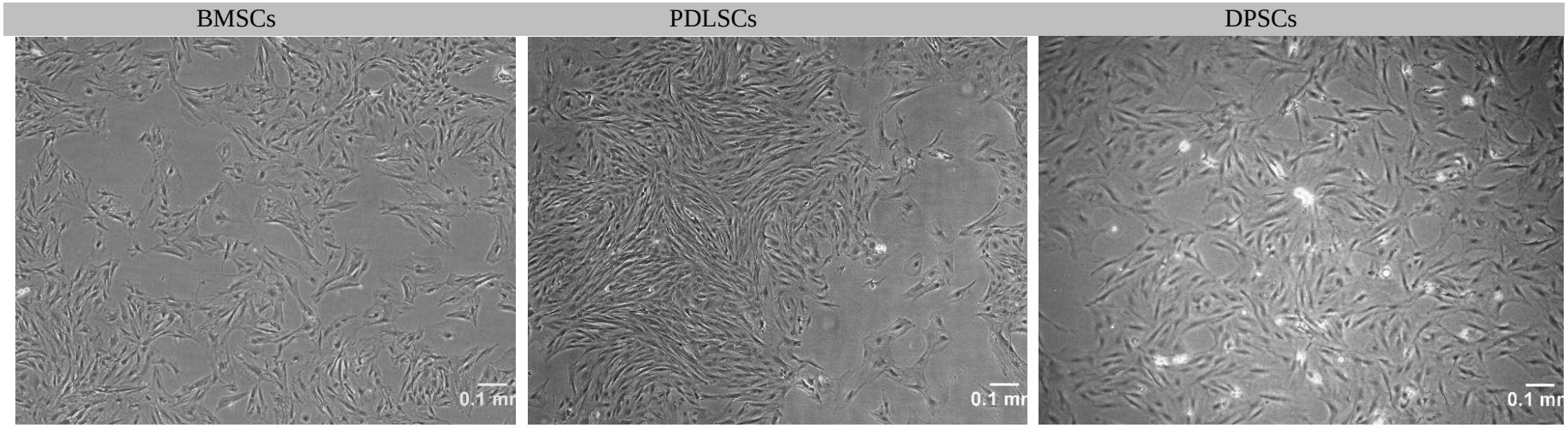


Figure 3.1 The appearance of the BMSCs, PDLSCs and DPSCs in passage 3.
Phase-contrast microscope images of the stem cells showing heterogenous morphology with spindle-like shaped cells at 10x magnification. Scale bars represent 0.1mm.

3.3 Identification of Stem Cell Markers

RT-PCR gene expression analysis of passage 3 cultures showed that BMSCs, PDLSCs and DPSCs expressed the genes for stem cell markers which were CD105 and CD90 (Dominici, Le Blanc et al. 2006). There was also expression of other suggested stem cell markers which are CD29, CD44 and c-myc. CD29 is also known as integrin-β1 and related to adipogenic progenitor cells (Davies, Cooper et al. 2015) ; CD44 is the cell surface adhesion molecule (Lesley, Hascall et al. 2000) and *c-myc* (Takahashi and Yamanaka 2006) were also expressed by all three cells.

The level of CD105, CD90 and CD29 expression in PDLSCs and DPSCs were significantly lower than BMSCs. Nevertheless, the level of CD44 expression was slightly higher for PDLSCs and DPSCs but the difference was not statistically significant. PDLSCs and DPSCs showed similar expression of the *c-myc* gene as shown in Figure 3.2.

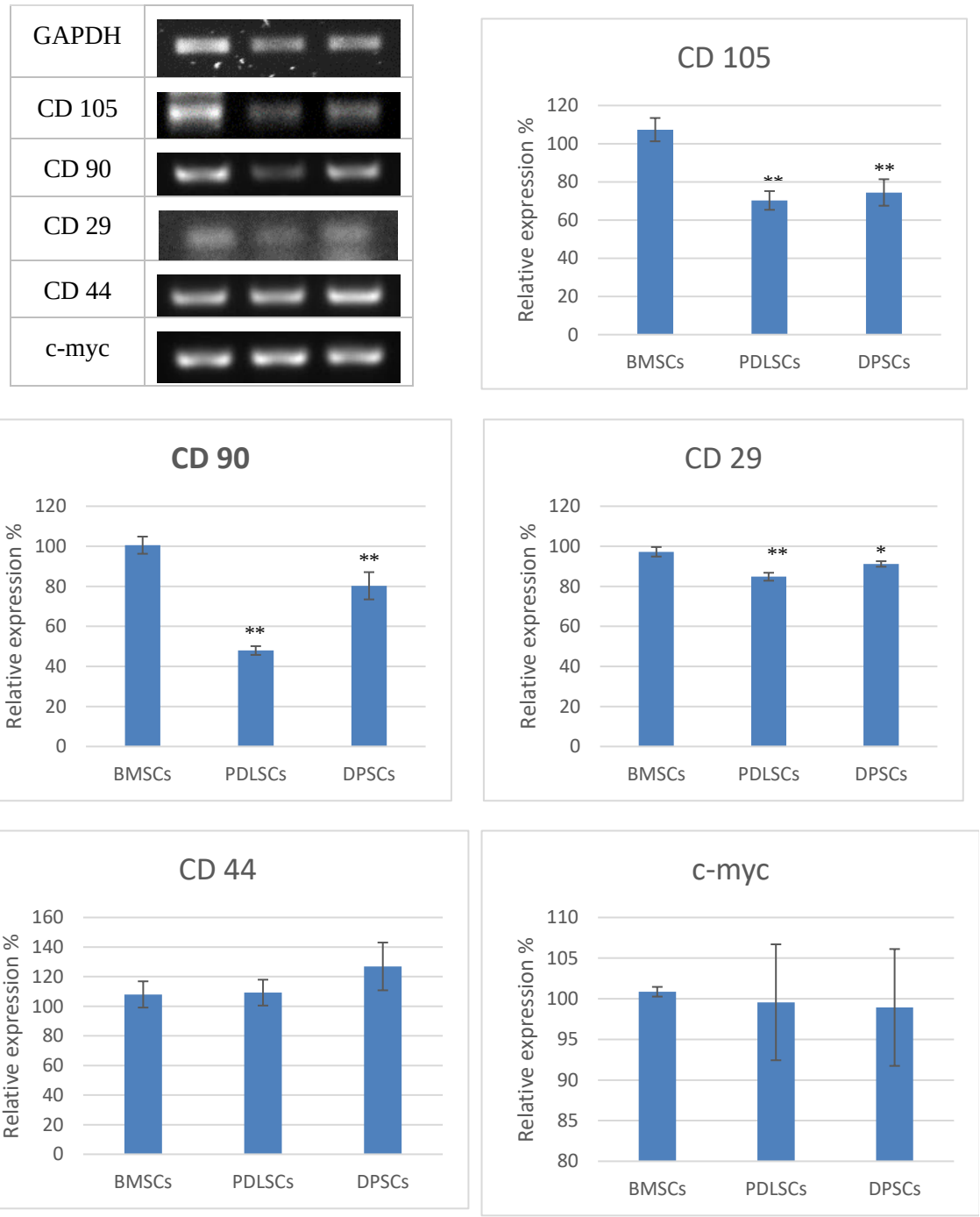


Figure 3.2 Stem Cell Markers

Semi-quantitative PCR analysis of gene expression profiles of BMSCs, PDLSCs and DPSCs showed all three cell-lines showed expression of the CD105, CD90, CD29, CD44 and c-myc. Y-axis is the relative expression to GAPDH. Statistical comparison was performed using BMSCs as control with *p-value<0.05 and **p-value<0.001, n=3 (technical replicates)

3.4 Osteogenic and Adipogenic Differentiation of Mesenchymal Stem Cells

Formation of calcified nodules is one of the end products for osteogenic differentiation of mesenchymal stem cells in culture. All three MCSs were cultured in osteogenic differentiation assay for 3 weeks with control groups cultured in 10% primary culture media to fulfil the recomendations by the ISCT that stem cells should be able to differentiate. BMSCs showed most prominent nodules which were highlighted with ARS. The ARS staining was also present for PDLSCs and DPSCs but not as much as in BMSCs which indicate lower osteogenic potential of PDLSCs and DPSCs compared to BMSCs. Control cells did not show any calcified nodules formation.

MSCs also showed signs of adipogenic differentiation assay once cultured in adipogenic differentiation media for 3 weeks. There was evidence of lipid containing cells among MSCs after stained with Oil Red O at the end of the differentiation assay (Figure 3.4). Control cells which were cultured in basal media consisted of α-MEM with 10% FBS were over-confluent and did not show any lipid containing cells. The findings were similar for all BMSCs, PDLSCs and DPSCs.

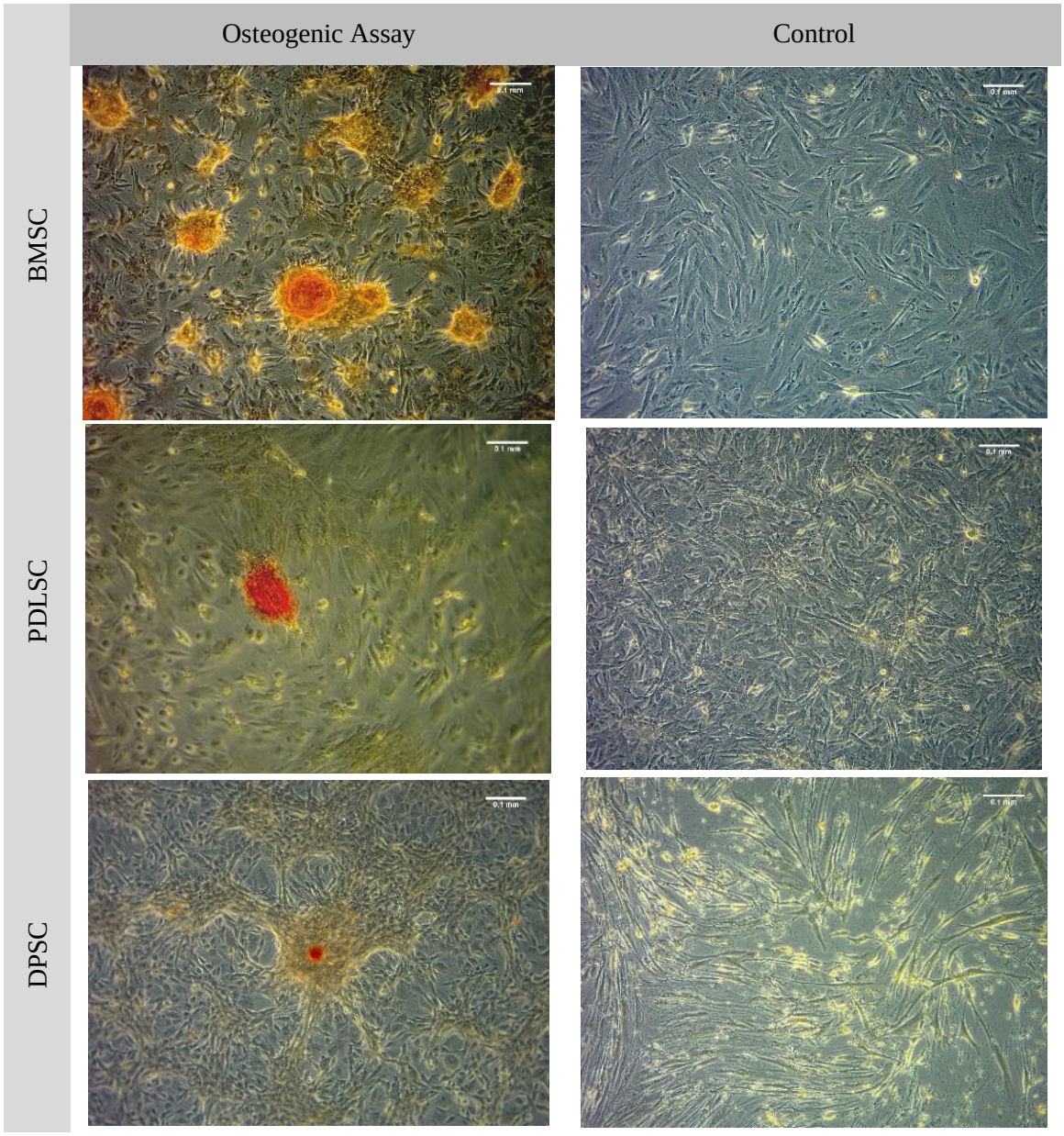


Figure 3.3 Osteogenic Differentiation Assay
Phase-contract microscope representatitive images of BMSCs, PDLSCs and DPSCs after osteogenic differentiation experiment. Alizarin red staining of the differentiated BMSCs, PDLSCs and DPSCs for mineralization showed formation of calcified nodules. Scale bar represent 0.1mm. The images of area with most abundance calcified nodul were presented.

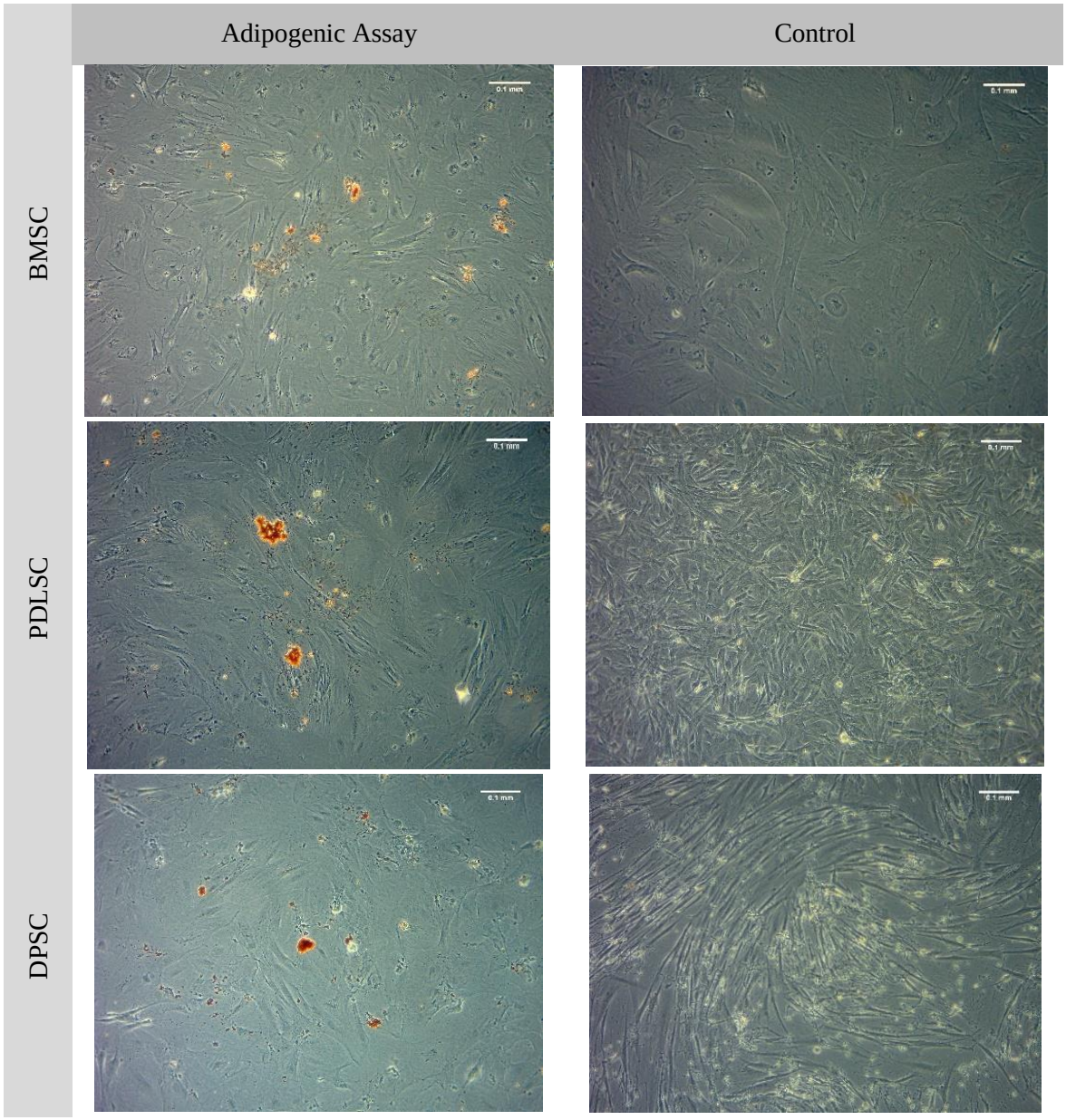


Figure 3.4 Adipogenic Differentiation Assay
Phase-contract microscope representatitive images of BMSCs, PDLSCs and DPSCs after adipogenic differentiation experiment. Oil red O staining of the differentiated BMSCs, PDLSCs and DPSCs demonstrated lipid-filled nodules. Scale bar represent 0.1mm.

3.5 Expression of osteogenic genes

One of the properties of the MSCs is the osteogenic potential. Thus, the expression of three osteogenic-related genes in PDLSCs and DPSCs in comparison to BMSCs, cultured in primary culture media were evaluated. The three genes were *Alkaline Phosphatase* (*ALP*), *Osteonectin* and *Core-binding factor alpha-1* (*cbfα-1*). Data showed DPSCs have similar pattern of osteogenic gene expression with BMSCs but PDLSCs expressed lower *ALP* and *cbfα-1*. The level of *osteonectin* gene expression was similar in all three cell-lines.

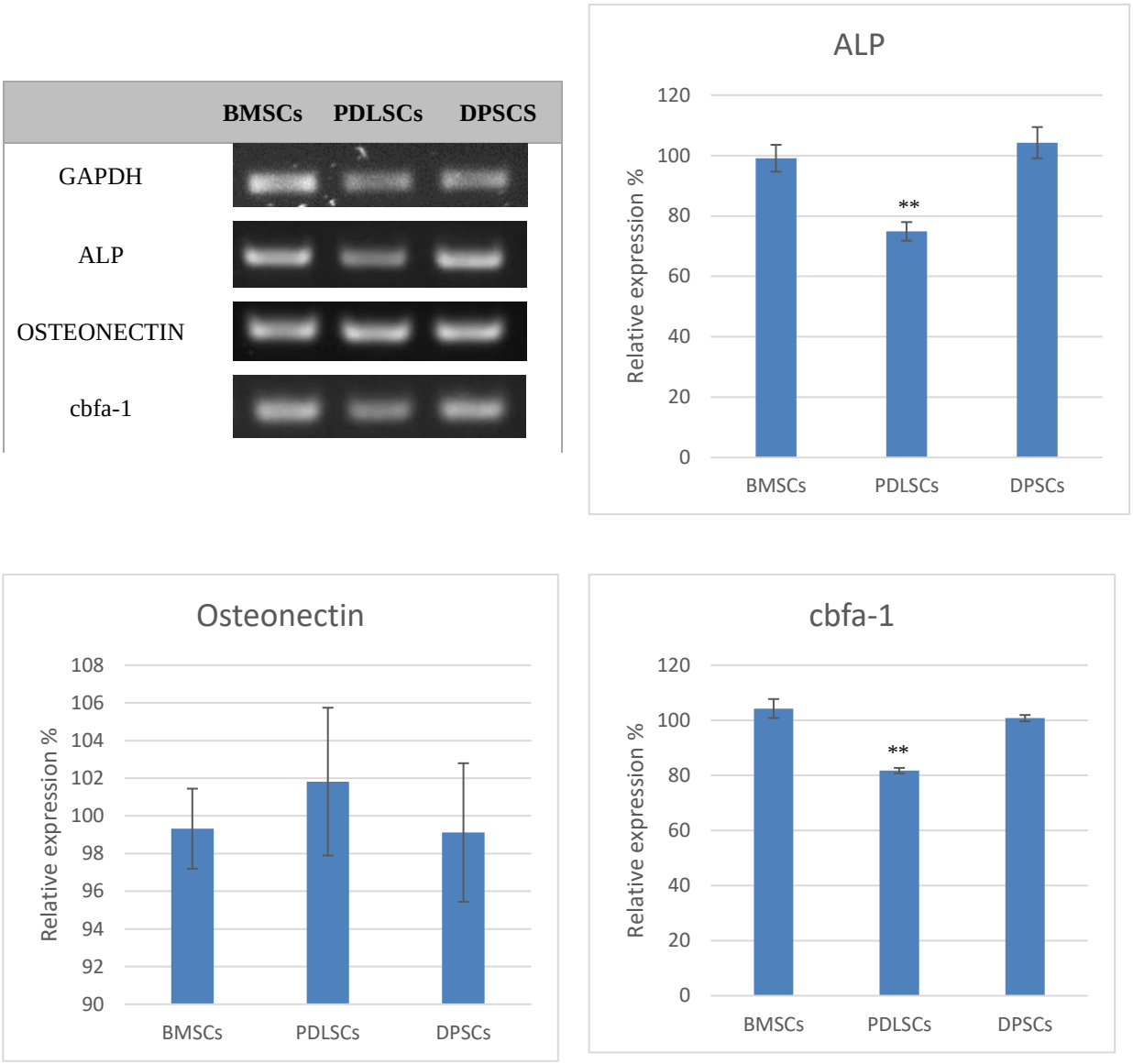
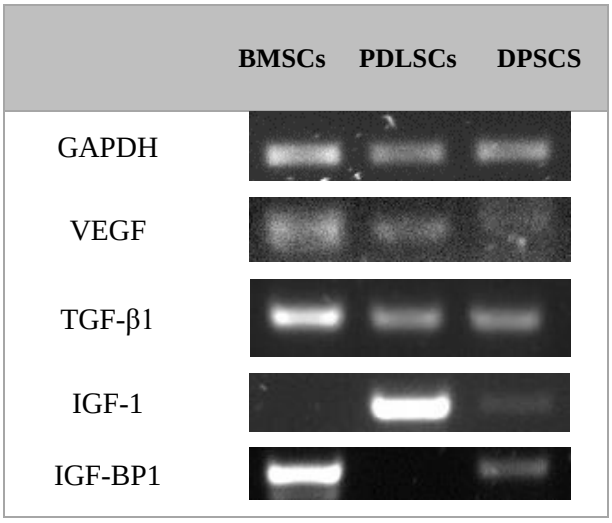


Figure 3.5 Osteogenic Gene Expression by the MSCs

Semi-quantitative PCR analysis of gene expression profiles of BMSCs, PDLSCs and DPSCs showed all three cell-lines showed expression of the osteogenic markers. Statistical comparison was performed using BMSCs as control with **p-value<0.001. n=3 technical replicates

3.6 Expression of growth factor genes

Analysis of the gene expression for the VEGF, TGF-β1 and IGF-1 was performed with semi-quantitative PCR. The cells were all cultured earlier in normal 10% FBS cell culture media. The gene expression for VEGF were almost similar between three cell-lines and the expression by BMSCs was the highest (Figure 3.6). Gene expression for TGF-β1 was also highly expressed by BMSCs and the expression were almost similar in PDLSCs and DPSCs. Interestingly, IGF-1 was highly expressed by PDLSCs whereas the expression by BMSCs and DPSCs was relatively low. In addition, the pattern was reversal for the IGF-BP1 gene expression which showed higher expression by the BMSCs and DPSCs but was undetectable in the PDLSCs.



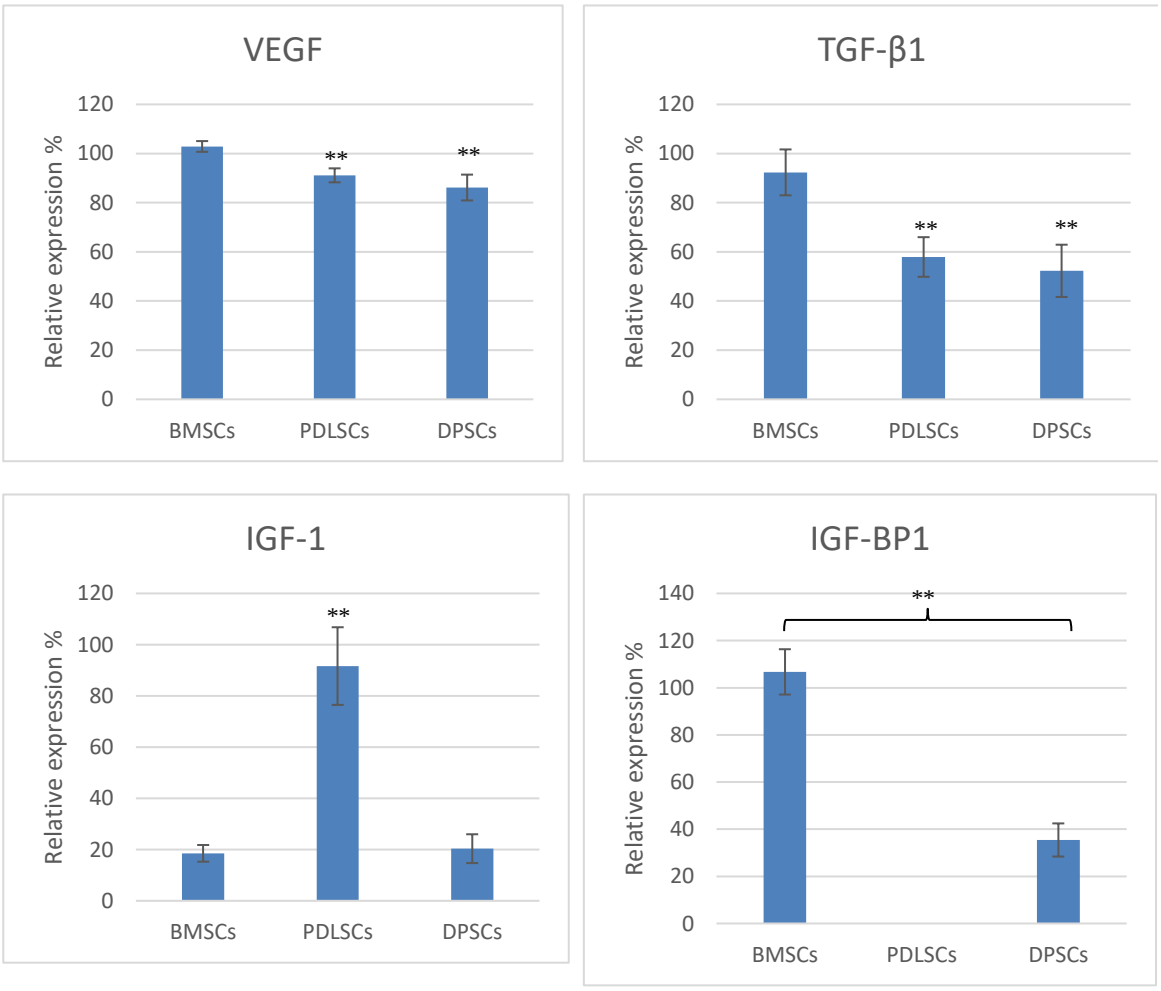


Figure 3.6 Growth factors gene expression of MSCs

Semi-quantitative PCR analysis of gene expression profiles of BMSCs, PDLSCs and DPSCs showed the expression of VEGF, TGF-β1, IGF-1 and IGF-BP1 of the three cell-lines. PDLSCs showed no expression of IGF-BP1. Statistical comparison was performed using BMSCs as control with **p-value<0.001, n=3 technical replicates

3.7 Discussion

Comparison studies of the different types of MSCs are more powerful if the the cells are isolated from same individual. This is to reduce the variability that may affect cell characterization and behaviour such as in gene expression and protein secretion (Paradisi, Alviano et al. 2014). In this study, rat mesenchynmal stem cells were used to enable comparison between cell types and the comparison could be performed with reduced inter-individual variabilities. The methods used for isolation of the MSCs were as described in previous reports (Davies OG 2014, Gao, Walmsley et al. 2016).

3.7.1 Growth Factors' gene expression

It was interesting finding to note that the expression of IGF-1 was relatively high only in the PDLSCs and almost not detected in the other two cell lines and the expression of IGF-BP1 was at relatively low levels in the PDLSCs sample compared to BMSCs and DPSCs. It is speculated that the high expression of IGF-1 in PDLSCs compared with other two cell types is probably due to high turnover rate of PDL cells which later form collagen (Skougaard, Levy et al. 1970, Orlowski 1978) and periodontal ligament half-lives have been reported to be within 2-6 days and the tissues are considerably metabolically active (Rippin 1976). Since IGF-1 were reported to have high affinity to other six binding proteins, the level of IGF-BP1, -2, -3, -4, -5 and -6 in PDLSC samples is worthy of further study. Currently, there are few studies reporting on which IGF-BPs bind to IGF-1 secreted by PDLSC, although result of microarray study showed the secretome of human PDLSCs contains relatively high level of IGF-BP6,-2 and -4 but lower levels of IGF-BP1 and -3 (Nagata, Iwasaki et al. 2017). The study also reported higher level of IGF-2 compared to IGF-1 in their PDLSC CM (Nagata, Iwasaki et al. 2017)

Low level of IGF-1 gene expression in the DPSCs sample is in line with other previous reports (Joseph, Savage et al. 1996, Alkharobi, Al-Khafaji et al. 2018). Indeed a recent report by Alkharobi

indicated increased of IGF-BP2 and IGF-BP3 gene expressions, however they did not report on the IGF-BP1 expression in their DPSCs (Alkharobi, Al-Khafaji et al. 2018)

As for the BMSCs, it was speculated that the low level of IGF-1 and high level of IGF-BP1 gene expression in BMSCs is probably due to the need to protect the IGF-1 from degradation in bone marrow niche and to deliver the IGF-1 produced by BMSCs to specific tissue (Youssef, Aboalola et al. 2017). Apart from IGF-BP1, other IGF-BPs were also found in CMs of BMSC such IGF-BP2,-3,-4 and -6 in their microarray profiles (Chen, Tredget et al. 2008, Ando, Matsubara et al. 2014) but specific roles of these IGF-BPs is not yet fully understood. Thus, the level and role of IGF-BPs secreted by the BMSCs is worthy to be studied further.

3.7.2 Osteogenic markers

There are several important genes related to osteogenic potential. *Cbfa-1* is the gene that regulates osteoblast differentiation and osteoblast functions (Karsenty 2000) and is present in any cell-lineage that produced mineralized tissue. Data here confirmed that this gene was expressed in all three cell types, however the expression of *cbfα-1* in the PDLSCs was lower than in the BMSCs which are known as the progenitors for osteoblasts. Notably, the anatomy of periodontal ligaments consists of fibers attached to the alveolar bone and cementum and no bony structure is expected to be in the PDL space. Consequently, the osteogenic potential of PDLSCs is likely lower compared with BMSCs and DPSCs and this is supported by our osteogenic differentiation assay which showed lower calcified nodules formation compared to the other two cell-lines.

A recent study has demonstrated the higher expression of *cbfa-1* in PDLSCs compared to DPSCs and BMSCs (Gay, Cavender et al. 2014). In that human study, different donors provide the source of each type of the cells specifically BMSCs. Thus, inter-individual's variabilities could be the reason for the result obtained such as age of the BMSCs donors and systemic disease that the donors probably have which alter the genetic profiles.

The *ALP* gene is another marker for osteogenesis (Choi, Noh et al. 2011) as ALP activity increases the inorganic phosphate during mineralization process (Golub and Boesze-Battaglia 2007). It is expressed higher in differentiating cells, usually after 7 days in osteogenic culture (Gay, Chen et al. 2007, Yu, He et al. 2010). Data presented here showed the level of ALP expression for BMSCs and DPSCs in normal growth medium were similar but slightly lower by the PDLSCs.

The expression of the *osteonectin* gene was also evaluated. Osteonectin or secreted protein acidic and rich in cysteine (SPARC) is a protein that binds to collagen through its c-terminal domain (Rosset and Bradshaw 2016). The higher level of *osteonectin* expressed by the PDLSCs in this study compared with the other two cell types may relate to the anatomy of the PDL where there is abundance of collagen fibers in the PDL spaces. Furthermore, osteonectin has been shown as one of the important proteins in areas with high collagen turnover such as in PDL. In one recent animal study using mice, the collagen fibrils in osteonectin/SPARC-depleted PDL was less thicker compared to the wild-type PDL (Trombetta, Bradshaw et al. 2010).

Pattern of calcific nodules formation was found to be varied between cell-types. According to Davies et.al, 2004, the pattern of mineralisation between DPSCs and BMSCs was different; the DPSCs formed more of a sheet-like mineralised matrix rather than being nodular in shapeas generated by the BMSCs (Davies, Cooper et al. 2015). In this study, BMSCs formed the most mineralization nodules and these numbers were the less in PDLSCs and DPSCs. The size of the mineralization nodules in PDLSCs and DPSCs were also smaller compared with the ones generated in BMSCs cultures.

Previous works with MSCs has been established from this research group, thus the multi-differentiation works were limited to the osteogenic and chondrogenic differentiation assays in order to focus on other important research works that suited the aim and objectives of the thesis. The same reason applied to the lack of negative markers for the MSCs (Davies OG 2014, Gao, Walmsley et al. 2016).

One of the limitations in this study is the usage of rat's incisors for DPSC isolation. This is because, rat's incisors are known to have open root and continuously growing compared to the molars. This may affect the expression of certain growth factors. Thus, other teeth such as premolars or molars should be used in future study to isolate both PDLSC and DPSCs for better comparison (Klevezal and Shchepotkin 2012).

Another aspect which should be discussed in this study, is the usage of semi-quantitative PCR technique in gene expression analysis between samples. Reference gene of the cells was used as internal control to quantify the gene expression. The internal reference genes used in this study is *GAPDH* and different cells expressed different level of *GAPDH* (Barber, Harmer et al. 2005). Thus, for better comparison between three different cell types, it is recommended to use absolute quantitative PCR in which the exact amount of gene expression based on calibrated standard curve.

3.8 Conclusion

Based on the data presented in this chapter, the isolation of the MSCs from three different sources were highlighted. The characteristics of mesenchymal stem cells as listed by ISCT in 2006 for BMSCs, PDLSCs and DPSCs were observed through their multidifferentiation potential and stem cell marker espression.

In conclusion, the characteristics of DPSCs were almost similar with BMSCs in terms of their secretion of growth factors and their osteogenic gene expression compared with PDLSCs.

CHAPTER 4

RESULT 2

Effect of Serum Concentration and Hypoxia Incubation on the Secretome Content

4 RESULT 2

4.1 Introduction

Secretomes of MSCs have been studied to evaluate its application in regenerative therapy. As the characteristics of three cell types have been recognized in previous chapter, focus of the study in this chapter are:

i. To compare the MSC cell growth in normal (10%) and low (0.5%) serum level culture media
ii. To compare the secretomes from the BMSCs, PDLSCs and DPSCs cultures under hypoxic (2% O_2) and normoxic (21% O_2) cultures.
iii. To compare the gene expressions of the BMSCs, PDLSCs and DPSCs between hypoxic and normoxic cultures.

The MSCs of passage 3 to 5 were seeded into 35mm dishes with cell density of 2.5 x 10^4 cells/ml with 10% FBS media. Half of the prepared wells were placed in normal oxygen (21%) incubation and another half were incubated in the hypoxia incubator (2%). The culture media in each well was refreshed every 3 days. Three wells of cell culture from each incubation were trypzinized and cell counts as in section 2.1.5 were performed to monitor cell growth. The cell counting was repeated every three days until the twelfth day. The experiments were performed four times.

The MSCs of passage 3 to 5 were also seeded into 35mm dishes with cell density of 2.5 x 10^4cells/ml with 10% FBS primary culture media and incubated either in normal oxygen (21%) or hypoxic incubations (2%). After 24 hours, the cells were divided into another two groups; 10% FBS and 0.5% media as detailed out in section 2.5. After three days, the CM were collected for analysis of growth factors secreted by the cells. The viable cells of each well were counted as describe in section 2.1.5.

The above experiment was repeated but the cells were cultured in serum free media for 3 days as detailed out in section 2.6. The conditioned media were collected for growth factor analysis and the cells were used for *VEGF, TGF-β, IGF-1* and *IGFBP-1* gene expression analysis as reported in section 2.2.

4.2 Proliferation of MSCs in Hypoxia Incubation and Normal Growth Medium (10% FBS)

The effects of the MSC cell growth on different oxygen concentrations were evaluated by culturing the MSCs in different oxygen environments: 21% oxygen (O_2) for normoxia and 2% O_2 for hypoxia incubations for 12 days. The results of the cell count at every three days time point showed that the BMSCs have higher proliferation in normoxic condition whilst DPSCs proliferate better in hypoxic condition. The growth of the PDLSCs were initially better in hypoxic but slowing down by Day 9 and there was evidence of dead cells which were stained by the Trypan blue dye by Day 12 in hypoxic environment (Figure 4.1).

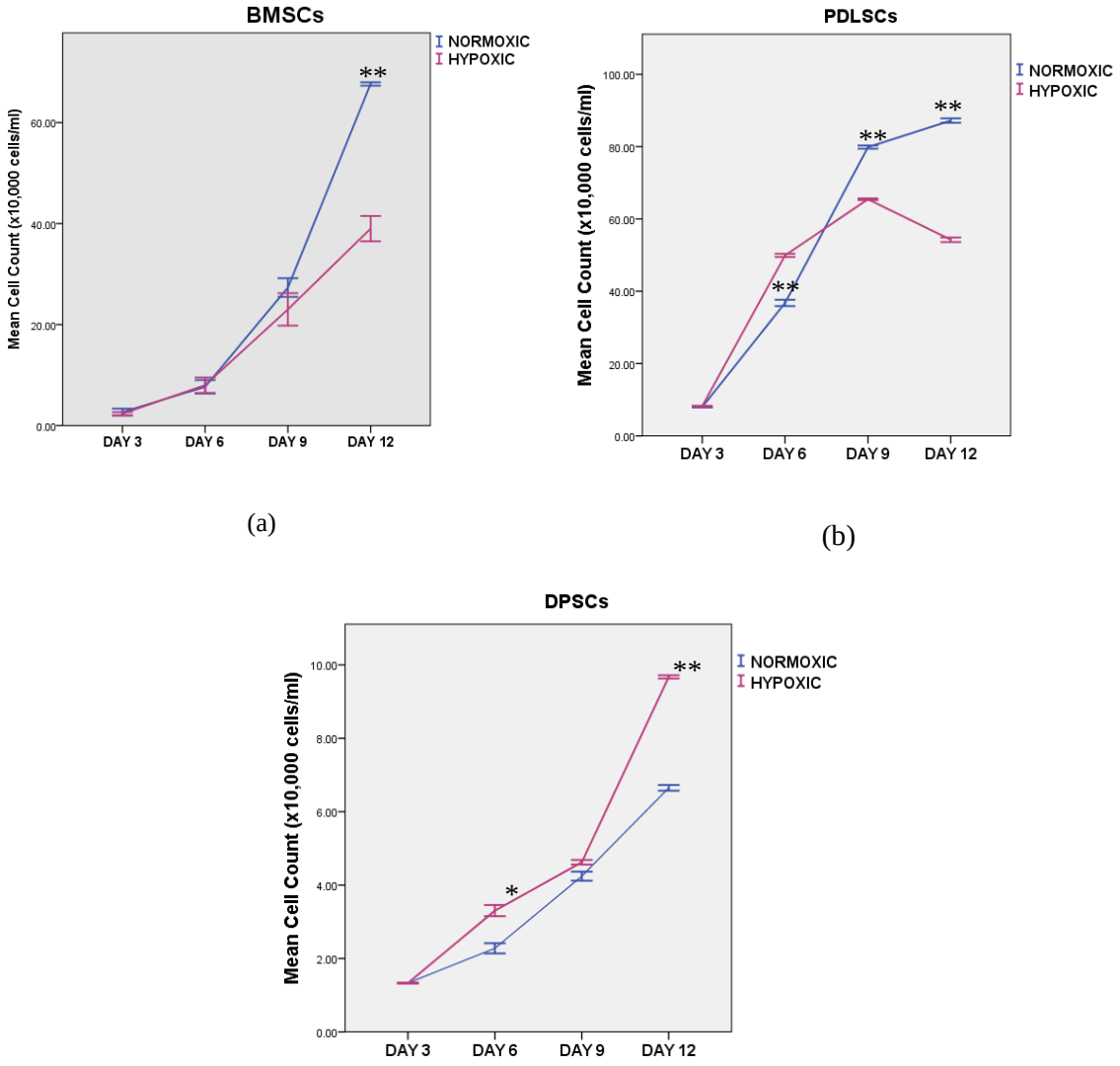


Figure 4.1 MSCs' growth in normoxia and hypoxia incubations

The cell count of the cells in each incubation environment were analysed and the statistical comparison was performed using normoxic cultured cells as control with *p -value<0.05 and **p-value<0.001. Value represents mean cell count in 4 experiments.

4.3 Level of growth factors secreted by mesenchymal stem cells

4.3.1 Different serum levels

The effects of the different serum concentration (10% FBS versus 0.5% FBS) on the MSCs cell growth and growth factors secreted by the cells were evaluated by culturing the MSCs with 10% or 0.5% FBS of primary culture media in standard oxygen environments (21% O_2) for three days.

The results confirmed that all three MSC types have significantly reduced cell expansion in low serum level (0.5%) compared to 10% FBS media. The secretion of the growth factors by the cells were also affected by the low serum level. There were significantly lower VEGF and TGF-B1 secretion in low serum level cultured cells compared to cells cultured in 10% FBS. However, the level for IGF-1 was significantly higher in low serum level (Figure 4.2). However, IGF-1 was not detected in the BMSCs and DPSCs' CM.

Similar findings were found when the cells were cultured in hypoxia (2% O_2). The cell number and growth factor secretion by the cells were lower in low serum level. Nonetheless, the level of IGF-1 secretion was also higher in low serum level cells as found in cells cultured in normoxia incubation (Figure 4.3). The secretion of IGF-1 was not detected in CM samples from BMSCs and DPSCs.

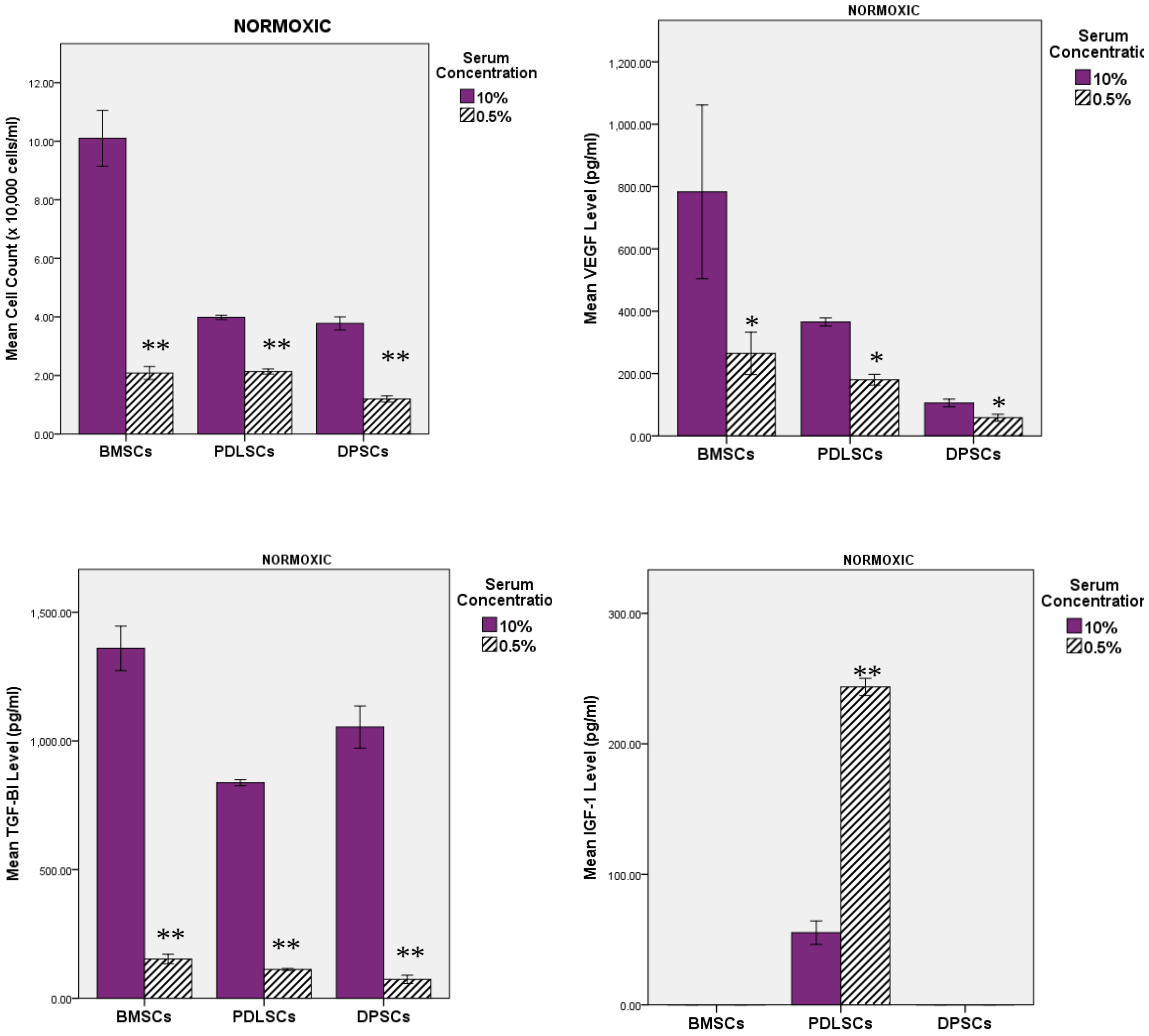


Figure 4.2 Growth factors production by MSCs cultured in <u>normoxia</u> incubation with different serum level

The cell growth of the MSCs after 3 days in 10% and 0.5% FBS and the level of collected growth factors in the culture media were analysed. The statistical comparison was performed using 10% FBS cultured cells as control group with *p -value<0.05 and **p-value<0.001. n=3 (technical replicates)

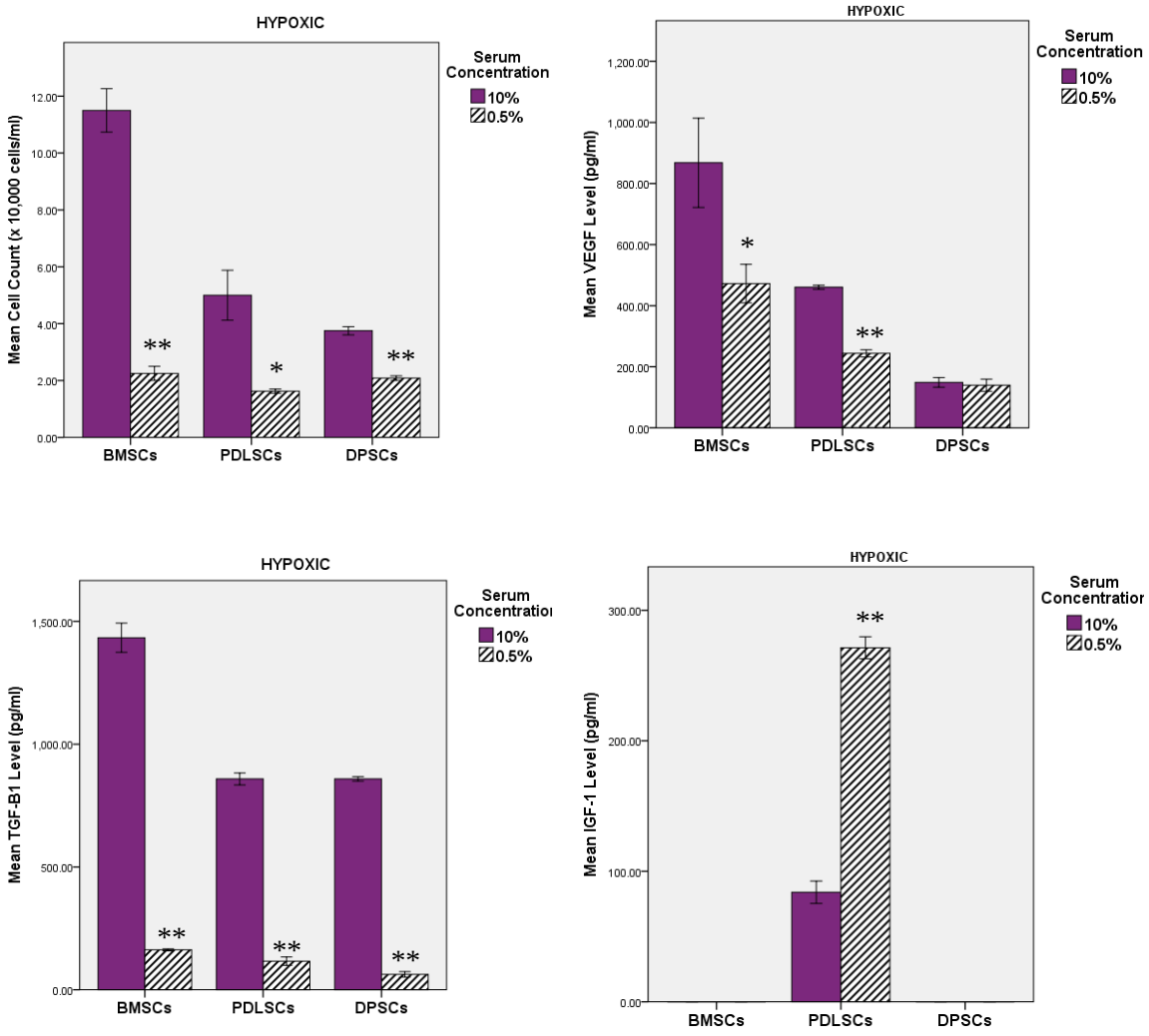


Figure 4.3 Growth factors production by MSCs in <u>hypoxia</u> incubation with different serum level

The cell growth after 3 days in 10% and 0.5% FBS in hypoxia (2%) incubation and the level of collected growth factors in the culture media were analysed. The statistical comparison was performed using 10% FBS cultured cells as control group with *p -value<0.05 and **p-value<0.001. n=3 (technical replicates)

For further analysis of the data, the level of growth factors was normalized to cell number of each culture conditions; to evaluate the expression of growth factors by individual cell of each cell line. The reason for this was to predict the amount of the growth factor produced if we were to collect the secretome in a larger amount for future experiments. In addition, this approach may eliminate other variable such as increase cell number due to favourable environments, which will also, caused increase level of secreted growth factors.

Results showed that hypoxic culture produced more VEGF in low serum concentration for all three cell-lines, but only PDL cells did not showed statistically significant difference. Even, there was no changes in VEGF production by PDLSC between 10% and 0.5% FBS when cells were cultured in standard culture condition (21%O_2). It showed that each PDLSC were not triggered to produce more VEGF once challenged with low serum and low oxygen as much as BMSCs and DPSCs. VEGF was secreted at higher level by BMSC and DPSC in low serum level of both culture conditions. The level of the growth factor has been corrected to the level of endogenous growth factors in the FBS.

The level of TGF-B1 was almost similar between normoxic and hypoxic culture. The findings also showed that only PDL cells secreted IGF-1 and the level was higher in hypoxic culture and lower serum ceoncentration.

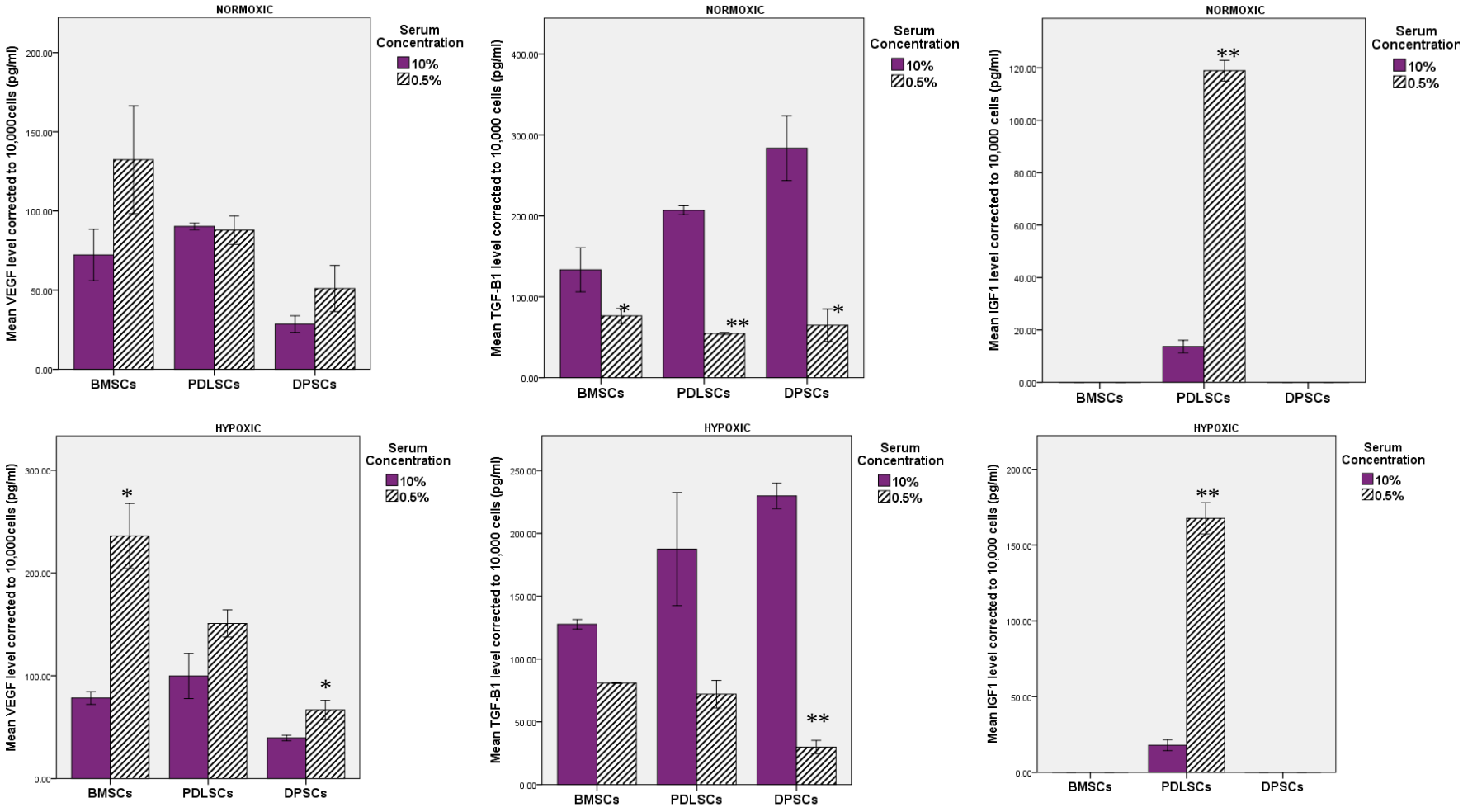


Figure 4.4 Growth factor production by MSCs corrected to 10,000 cells

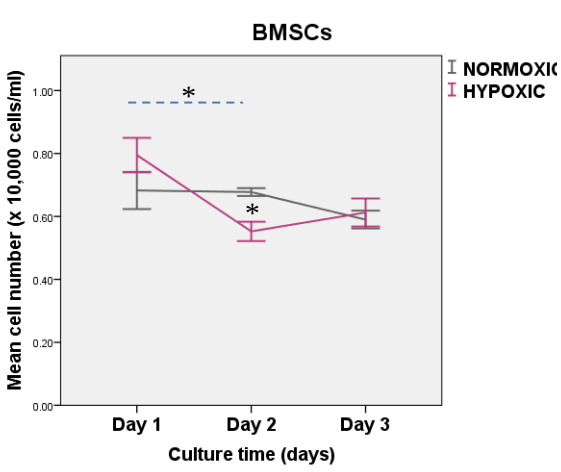
The level of growth factors released by the cell after 3 days in 10% and 0.5% FBS in hypoxia (2%) incubation were analysed. Statistical comparison was performed using 10% FBS group as control with *p -value<0.05 and **p-value<0.001. n=3 n=3 (technical replicates)

4.4 Proliferation of the MSCs in serum-free media

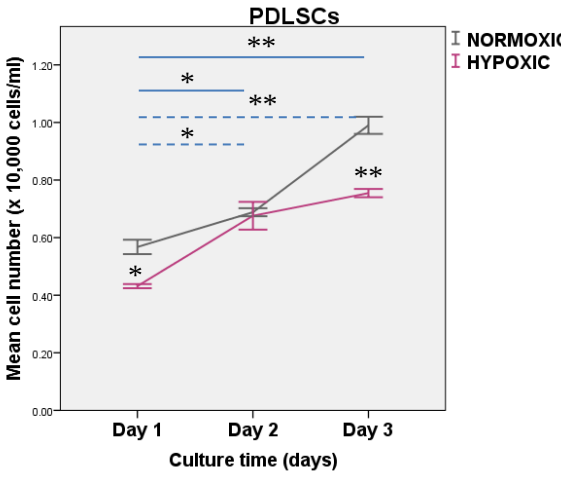
As the BMSCs, PDLSCs and DPSCs showed cell growth after 3 days despite less serum level in section 4.3, their cell growth was evaluated further by cell count in total serum-free media for three days in both 2% and 21% oxygen concentration.

The results showed that when the cells were cultured in serum-free media, there were no obvious difference between different oxygen incubations to the cell number up to third day except for PDLSCs where the cell numbers were significantly reduced in hypoxia incubation. The trends of the cell growth of BMSCs, PDLSCs and DPSCs were different on each day point between two oxygen incubations.

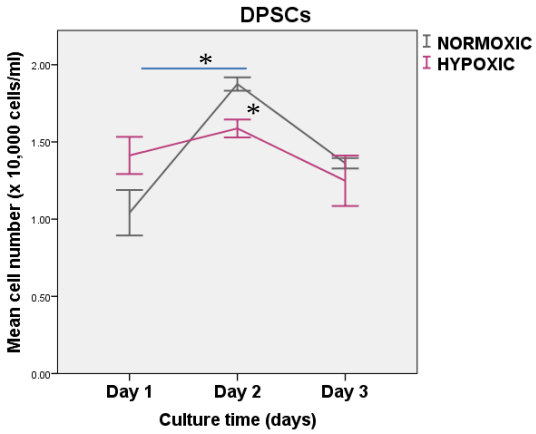
Analysis of the cell growth between days, showed no statistically significant difference in cell numbers between day 1-2 and day 1-3 in BMSCs and DPSCs incubated in normoxia nor hypoxia except the one showed in Figure 4.5(a) and (c). However, increased cell numbers were observed at Day 2 and Day 3 of PDLSCs incubated in both normoxia and hypoxia incubations and the increments were statistically significant (Figure 4.5 (b)).



(a)



(b)



(c)

Figure 4.5 Proliferation of the MSCs in serum free media and different oxygen concentration Relative cell numbers of both cells were used. The statistical analysis was performed in two ways: 1) between normoxic and hypoxic cells at every time point and 2) between day 1 and day 2 or day 3. Analysis between days was performed using Day 1 as control. n=5 (technical replicates)

Comparison between normoxic cells

Comparison between hypoxic cells

4.5 Level of growth factors secreted by mesenchymal stem cells cultured in serum-free media

VEGF secretion was significantly greater by the hypoxic-incubated cells compared to normoxic-incubated cells of BMSCs, PDLSCs and DPSCs. The level of TGF-B1 was only statistically higher in hypoxic cells of PDLSCs and DPSCs. As previous findings in section 4.3, IGF-1 was only detected in PDLSC cultures and not the other two cells. The hypoxic-incubated cells produced higher IGF-1 compared to normoxic-incubated cells (Figure 4.6).

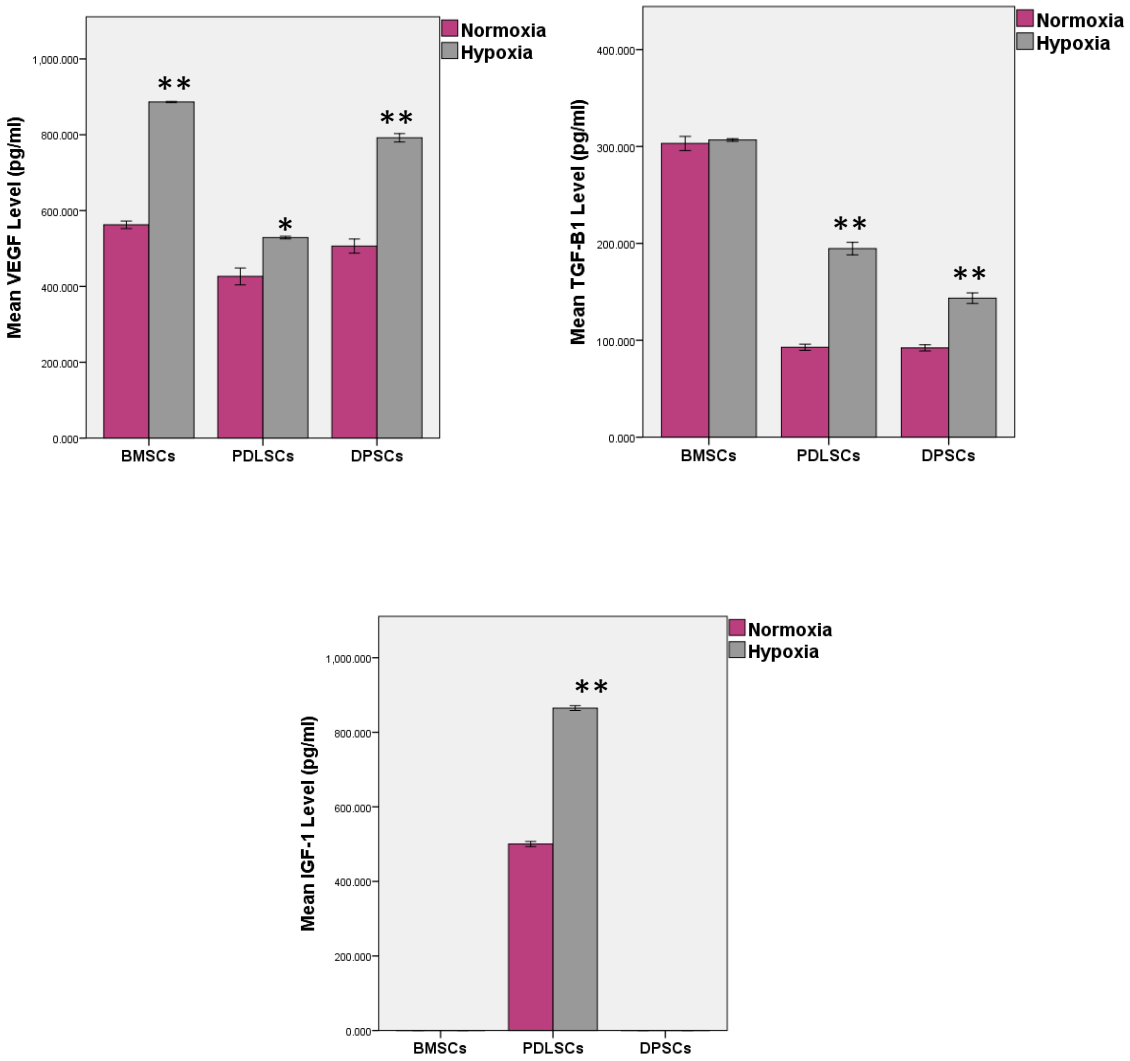


Figure 4.6 Growth Factor production by the MSCs in serum free media culture

The level of growth factors released by the cell after 3 days in serum-free media and different oxygen incubations. Statistical comparison was performed using normoxic cultured cells as control with *p -value<0.05 and **p-value<0.001. n=3 (technical replicates)

4.6 Growth factor gene expression in normoxia and hypoxia cultured cells

The gene expression of the three growth factors which were VEGF, TGF-β1 and IGF-1 as reported in section 3.6 were analysed between normic and hypoxic cultured cells.

There were no significant differences in *VEGF* and *TGF-B1* expressions of all three cell types although the expression of VEGF by BMSCs normoxia samples appeared higher than hypoxia samples. BMSC and PDLSC hypoxic cultures expressed higher *IGF-1* and conversely, normoxic samples of DPSCs expressed higher *IGF-1* despite very low detection in BMSCs and DPSCs (Figure 4.7 and Figure 4.8).

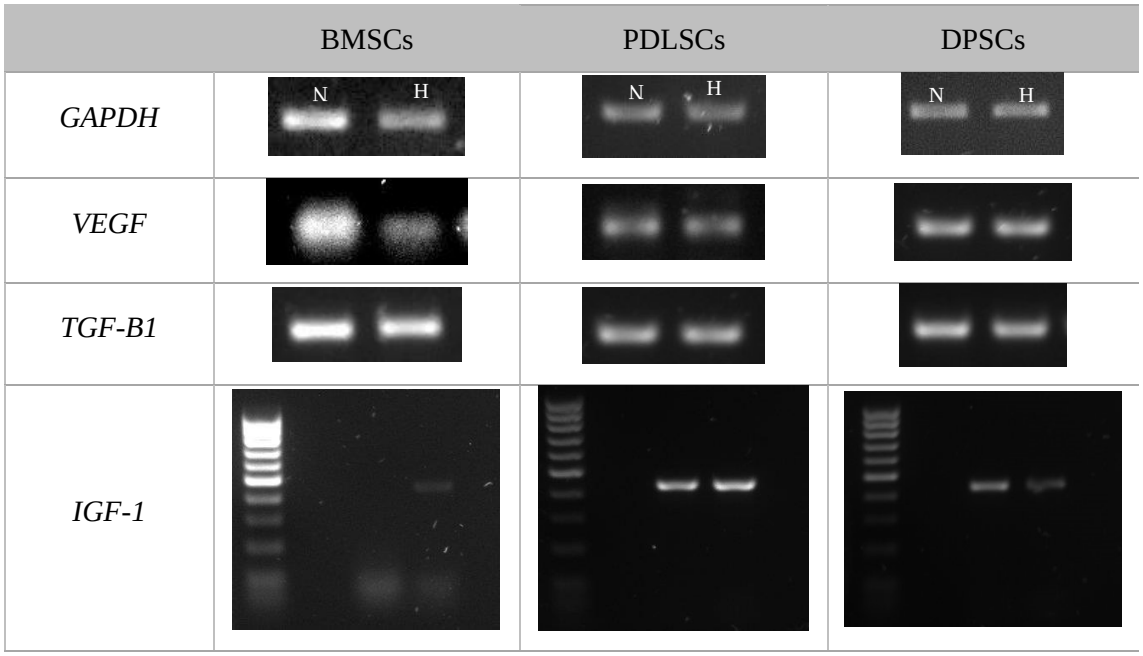


Figure 4.7 Growth factors gene expression gel images between normoxia and hypoxia cultured cells

Semi-quantitative PCR analysis of gene expression profiles of BMSCs, PDLSCs and DPSCs in different oxygen incubation showed the expression of VEGF, TGF-β1 and IGF-1 of the three cell-lines. Statistical comparison as shown in Figure 4.8

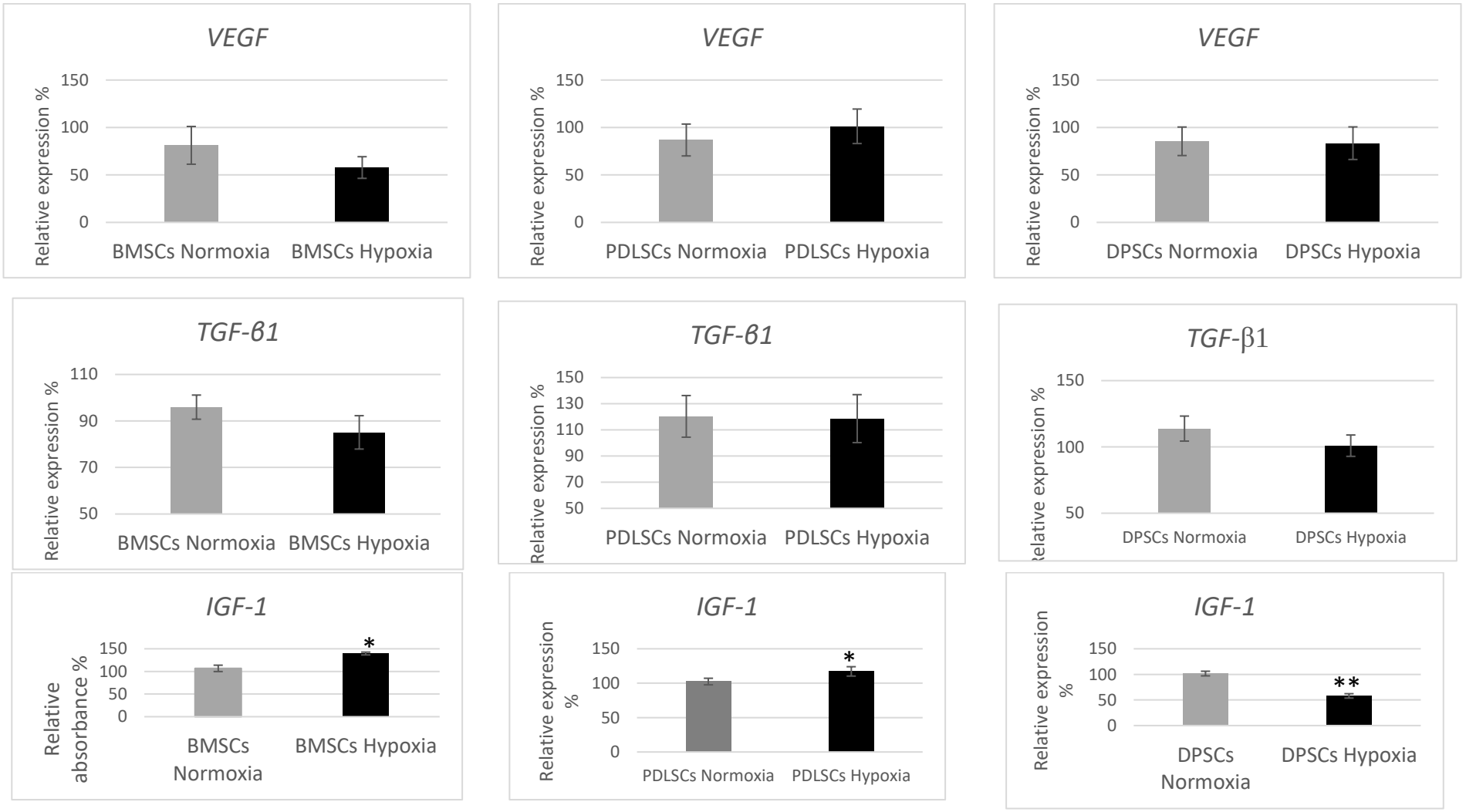


Figure 4.8 Growth factors gene expression by all MSC cultured in normoxia and hypoxia

Semi-quantitative PCR analysis of gene expression profiles of BMSCs, PDLSCs and DPSCs showed the expression of VEGF, TGF-β1 and IGF-1 between normoxia and hypoxia cultures. Statistical comparison was performed using normoxic cultured cells as control with *p-value <0.05 and **p-value<0.001 n=3 (technical replicates)

4.7 Discussion

4.7.1 Stem cells proliferation in hypoxia and lower serum level

In this study, cell number was assessed at certain culture time points to determine cell expansion or cell growth in different culture conditions. Hypoxia- stimulated proliferation of MSCs have been shown in previous studies (Lennon, Edmison et al. 2001, D'Ippolito, Diabira et al. 2006, Grayson 2007). Furthermore, a reduced oxygen environment has been suggested to maintain the stemness of MSCs by maintaining the undifferentiated state of the cells; on the other hand increases in oxygen concentration would promote differentiation of MSCs (D'Ippolito, Diabira et al. 2006). Hypoxia also enabled BMSCs to maintain their undifferentiated state until later passages compared to cells cultured in 20% oxygen (Grayson 2007). Despite the findings in other studies, the growth curve of BMSCs in this study was similar in normoxia and hypoxia with normal serum level at relatively early time-points (up to 6 days) but proliferated better in normoxic environment in later culture time-points.

Conversely, PDLSCs proliferated better in hypoxic environment at the beginning of the culture period but cell growth decreased by ninth day due to cell death. Studies showed that the PDLSCs proliferation in 2% oxygen incubation was increased compared to the one cultured in normal oxygen concentration after 24 hours (Wu, Yang et al. 2013) and 7 days (Zhang, Zhang et al. 2014). Progenitor cells residing in periodontal ligament noramlly have a reduced oxygen environment as one *in vivo* study has shown that root development of a tooth in rats occurred in low oxygen concentrations (Choi, Jin et al. 2014). There is also one previous study which contradicts these results when the PDLSCs were cultured in 2% hypoxia incubation. The proliferation of the PDLSCs were slightly higher in normoxia incubation, however the difference was not significant (Zhou, Fan et al. 2014). The researchers also found that the effect of hypoxia incubation on PDLSC caused increased osteogenic, adipogenic and chondrogenic differentiation compared with cells cultured in normal oxygen concentration (Zhou, Fan et al. 2014).

This study also found that DPSCs proliferated better under hypoxia condition. This finding is consistent with other studies using both animal and human samples (Amemiya, Kaneko et al. 2003, Sakdee, White et al. 2009). DPSCs from dogs were used and the proliferation of DPSCs of the hypoxia group were greater than re-oxygenated cells. The cells of re-oxygenated group were incubated with 20% oxygen after 24 hours of reduced oxygen incubation. The other study incubated human DPSCs in 3% oxygen and the results showed that the proliferation of the cells were increased compared to cells incubated in normoxia cultures (Sakdee, White et al. 2009). However, these two studies did not compare the cell proliferation in different serum concentrations instead 10% FBS was used as their primary culture medium (Amemiya, Kaneko et al. 2003, Sakdee, White et al. 2009)

Another aspect of our study was to evaluate the effect of serum concentration on the level of growth factors in the CM. Although serum is important in any tissue culture procedure as it provides growth factors and nutrients to the cells, reduction or total elimination of serum proteins may enable the researchers to detect and identify secreted proteins which are usually secreted in very low amounts (Kupcova Skalnikova 2013). Generally, cells growth was reduced in lower serum level underscoring the role of serum, indicating specifically that certain growth factors promote cell proliferation.

This indicated that despite the fact the serum is crucial for cell growth; the cells probably have produced enough paracrine factors that enable them to survive. The ability of the MSCs to maintain their proliferation under low oxygen and low serum concentration may be due to the production of certain growth factors into their surrounding environment (D'Ippolito, Diabira et al. 2006, Oskowitz, McFerrin et al. 2011). This notion was supported by the observed increase in production of the growth factors VEGF by all cell types and IGF-1 by PDLSCs when cultured in lower serum level as is presented in section 4.3.1

When the cells were cultured in serum free media for up to three days, there were evidence of deteriorating cell viability after 2 days of culture for BMSCs and after 1 day culture for DPSCs; both cultured in normoxia and hypoxia (Figure 4.5) however this evidence was not apparent for PDLSCs. This is probably due to the presence of IGF-1 as anti-apoptotic factors in PDLSCs culture. Indeed

there iss evidence stating that there is epigenetic programming of the IGF-1 gene in MSCs once cultured in serum-deprived conditions and IGF-1 depleted CM showed higher cell apoptosis compared with cells cultured in non-depleted IGF-1 CM (Sanchez, Oskowitz et al. 2009).

4.7.2 VEGF production in the conditioned media

Studies have shown that VEGF production was higher by PDLSCs and DPSCs incubated in hypoxia with a 1-2% oxygen concentrations (Amemiya, Kaneko et al. 2003, Aranha, Zhang et al. 2010, Wu, Yang et al. 2013). In this study, VEGF was secreted at highest level by BMSCs in both hypoxia and normoxia after three days of culture whereas DPSCs' CM contained the lowest VEGF levels in both normoxia and hypoxia. The amount of VEGF levels was related to the number of cells in the culture, i.e. with increased number of cells in the culture, there were also increased production of the growth factors.

Thus, further analysis showed that each MSC type secreted different relative amounts of VEGF under different culture conditions. The highest level of VEGF per cell was actually secreted in hypoxic conditions with lowest serum concentrations noted for BMSCs. For PDLSC and DPSC, similar patterns were also observed however the levels of VEGF were lower than those produced by BMSCs. When the cells were cultured in serum-free media, VEGF was produced at higher levels in hypoxia for all three cell-types. Although this was not supported by the VEGF gene expression as there was no significant difference between normoxia and hypoxia samples for all three cell types. This may be due to post-transcriptional modification that occurred to the *VEGF* gene (Yoo, Mulkeen et al. 2006).

The relative high production of VEGF by BMSCs compared with DPSCs could probably reflect the source of the cells as bone marrow is a known source for progenitor cells for endothelial cells, not only under normal physiology but in any pathological conditions in the body (Asahara, Masuda et al. 1999).

Analysis of the trend of the results indicates that the culture conditions that produced highest VEGF level per cell apart from hypoxia was minimal serum concentration. The influence of serum deprivation is probably more important rather than the reduction of oxygen for production of VEGF as the pattern of increment in VEGF production with reduction of serum concentration can also be observed in normoxic cells. These findings may suggest that with less nutrients, the cell was stimulated to produce more growth factor for their growth and survival. This also means that that VEGF may have a protective role for the MSCs (Yalvaç, Yarat et al. 2013) as it promotes angiogenesis in order to provide more nutrients to the cells. There has been no other study evaluating the effect of low serum concentration on VEGF production involving dental stem cells.

4.7.3 TGF-B1 production in the conditioned media

TGF-B1 is considered an important growth factor in the tissue repair process following injury (Melin, Joffre-Romeas et al. 2000) and in cell differentiation (Melin, Joffre-Romeas et al. 2000, He, Yu et al. 2008). In this study, TGF-B1 was highly produced by each cell of DPSC compared with BMSCs and PDLSCs. The level of this growth factor produced by DPSC and PDLSC was higher in high oxygen concentration which implies that oxygen is important in the production of TGF-B1 by DPSC and PDLSC. The relatively high level of TGF-β1 in higher serum concentration cultures may be because TGF-β1 is present in the serum. There was actually no difference of TGF-B1 production by BMSC between different oxygen concentrations and proven in gene expression of TGF-β1. This indicated that oxygen is not a crucial element for TGF-B1 production by BMSCs. The role of TGF-B1 by DPSCs and PDLSCs was probably more important for recruitment and differentiation of cells during repair process for example in pulp and periodontal injury, but the role of this growth factor in bone marrow is more for maintaining cell proliferation (Zhang, Ren et al. 2015).

4.7.4 IGF-1 production in the conditioned media

An interesting finding of this study was that IGF-1 was only detected in CM from PDLSCs and not in CM from BMSCs and DPSCs. These findings were consistent with the gene expression profile as

determined by RT-PCR. In fact, the contrast of the PCR gel images had to be increased in order to detect the minimal amout of expression in BMSCs and DPSCs samples. This result corroborates findings in chapter 3 where it was found that the expression of IGF-1 was only detected in PDLSCs and not in BMSCs and DPSCs. Furthermore, the IGFBP-1 gene expression was also detected as being higher in BMSCs and DPSCs samples. The presence of IGFBP-1 probably caused the lower detection of IGF-1 as the protein molecules were bound to the binding protein. Conversely, IGF-1 is one of the growth factors involved in cell metabolism and regulates oxidative stress resistance (Holzenberger, Dupont et al. 2002), thus it suits the demand for the PDL cells as these cells have the fastest turn-over rate in the body (Rippin 1976).

4.8 Conclusion

In conclusion, there was a distinct growth pattern of PDLSCs compared with BMSCs as the control group in serum-free media and hypoxia conditions. The secretome from PDLSCs was also different compared with the other two cell-types, in particular due to obvious production of IGF-1 into the culture media. It can also be concluded that hypoxia incubation in general may be an advantageous condition for cell culture in order to generate optimum VEGF and IGF-1 in the secretome.

CHAPTER 5

RESULT 3

Variablities in conditioned medium preparation affect the secretome production

5 RESULT 3

5.1 Introduction

Apart from different oxygen concentrations, there are other factors that could alter the quantity of the protein content in the collected culture media. The focus of this chapter is:

i. To evaluate the effect of days of collection and filtration of the CM on the level of growth factors in the CM from the BMSCs, PDLSCs and DPSCs cultures.
ii. To evaluate the proteomic profiles of all CM samples via mass spectrometry.

In general, the CM was collected after the cells (BMSCs, PDLSCs and DPSCSs) were cultured with 10% serum media in T75 flask until 70-80% confluent before the media were changed to SFM and cells were incubated in either normal incubator with 21% oxygen, or in the hypoxic incubator in which the oxygen concentration was set to 2%. The CM were then collected from both normal and hypoxic incubators on second and third day of culture and kept in -20°C until used.

The collected CMs from different culture times were pooled together prior to the proteomic analysis such as ELISA and mass-spectrometry.

5.2 Variabilities in secretome production

5.2.1 Effect of Different Days of Collection

Cells were cultured in primary culture media until 80% confluent before changed to serum free media The CM were collected after 2 or 3 days and analysed for growth factors VEGF, TGF-β1 and IGF-1.

The results showed that for all samples from different cell types and different incubation types, the levels of VEGF and TGF-β1 were higher in day 3 samples compared to day 2 samples (Figure 5.1).

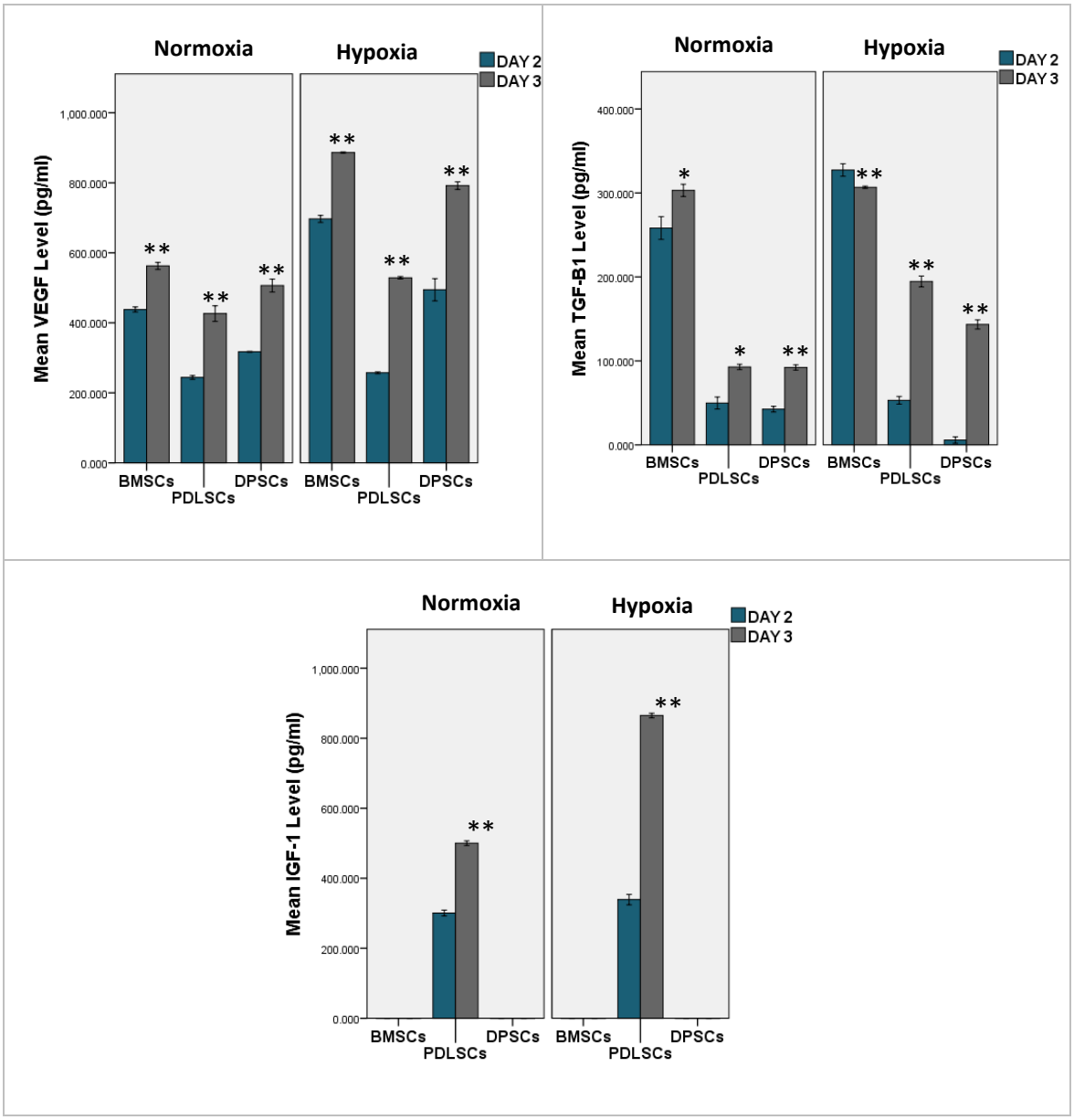


Figure 5.1 Growth factors levels in CM collected on different days.

The BMSCs, PDLSCs and DPSCs cultured for two and three days and the level of collected growth factors in the culture media were analysed. The statistical comparison was performed using Day 2 CM as control group with *p -value<0.05 and **p-value<0.001. n=3 (technical replicates)

5.2.2 Effect of CM Filtration

The CM were collected and divided into 2 groups. In one group, the CM were filtered with sterile-filtration membrane 0.2µm as sterilisation method and to remove any cell debris in the CM and in the other group, the CM were not filtered. The level of the three growth factors were analysed with ELISA kit. The results showed that filtrated CM contained significantly lower level of all three growth factors as presented in Figure 5.2 except for VEGF in BMSCs hypoxia samples (p-value = 0.81) and TGF-β1 in DPSCs hypoxia samples (p-value = 0. 215).

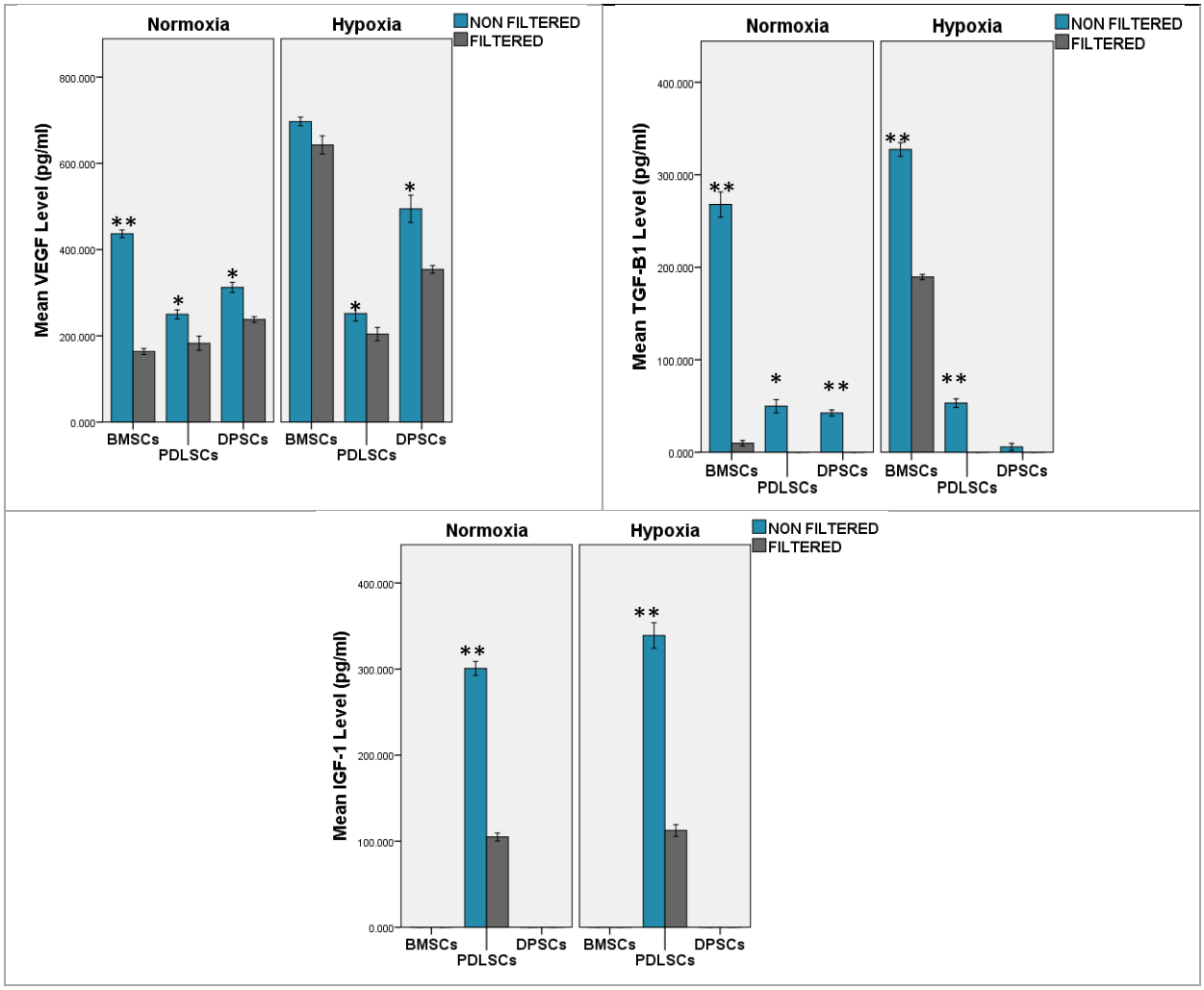


Figure 5.2 Effect of the filtration on the level of the growth factors in the collected CM. Level of VEGF, TGF-β1 and IGF-1 secreted by the BMSCs, PDLSCs and DPSCs with and without filtration with 0.2µm. Statistical comparison was performed using filtered samples as control with *p-value<0.05 and **p-value<0.001. n=3 (technical replicates)

5.3 Ultrafiltration of the CM

Result of section 5.2.2 showed that level of all three gowth factors were reduced in filtred samples. Thus, we speculated that some of the growth factors probably adhered to the filter membranes.

In order to evaluate the proteomic profiles of each CM samples via mass spectrometry analysis, the Day 3 CM were ultrafiltrated with 3kDa membrane for 30 minutes at 14000 rcf to produce ~ 10x concentrated CM based on manufacturer's instructions. For every 500µl of collected CM, around 55µl of concentrated CM obtained. The protein content in the concentrated CM was measured using BCA Assay.

5.3.1 Protein level of the concentrated CM

Results of the protein quantification showed that there was no significant difference between normoxia and hypoxia sample in all 10x concentrated CM samples. The statistical analysis was based on three experimental replicates as shown in Figure 5.3.

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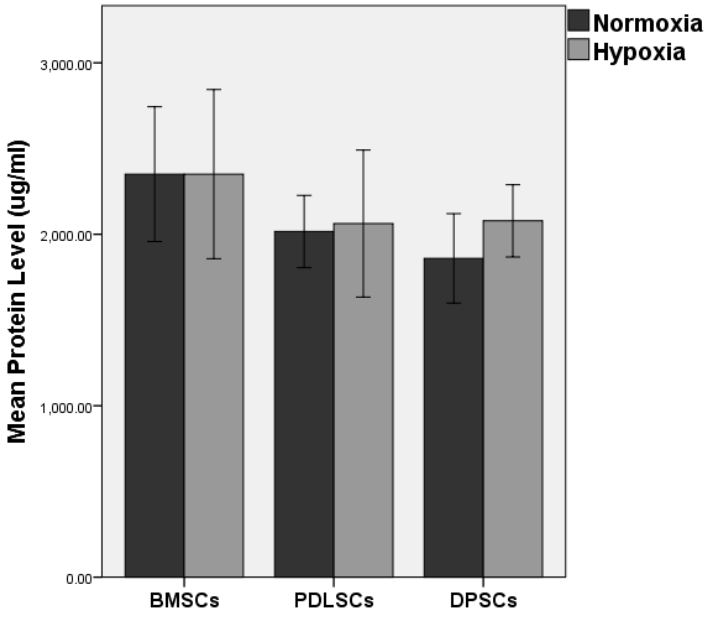


Figure 5.3 Protein level in the CM samples

The level of the protein in concentrated CM were analysed and statistical comparison was performed using normoxia samples as control.

5.4 Mass spectrometry analysis

The first part of mass spectrometry analysis was the qualitative analysis of three CM samples from three cell types of normoxic cultures. There were a total of 87 proteins detected in BMSCs sample, 53 proteins in PDLSCs and 79 proteins in DPSCs (Appendix 1). All samples were corrected to the control/blank samples ie; all proteins detected in serum free culture media have been eliminated from the list of proteins of each CM. Thus, all proteins listed from each CM samples were exclusively produced by the cells.

From all the detected proteins, 29 proteins were in common to all 3 samples (Table 5-1). BMSCs and DPSCs have more proteins in common compared to with PDLSCs as shown in Figure 5.4.

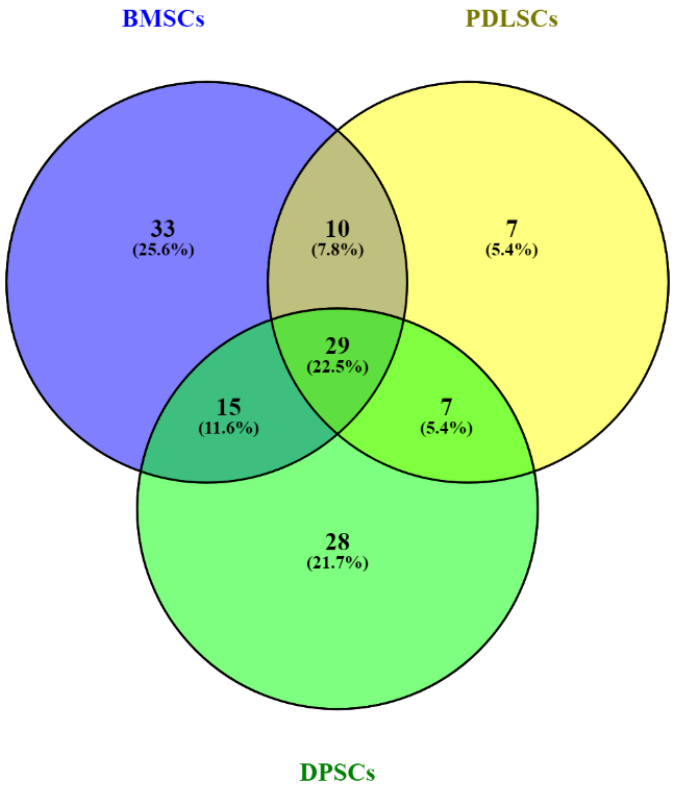


Figure 5.4 Venn diagram showing the detected proteins in CM samples.

Accession Number	Protein Names
Q7TP84	Plasminogen
Q6GMN8	Actn1 protein
A0A0G2K013	Alpha actinin 4
P07151	Beta-2 microglobulin
P47853	Biglycan
P00787	Cathepsin B
P07154	Cathepsin L1
P02454	Collagen alpha-1(I) chain
P13941	Collagen alpha-1(III) chain
P20909	Collagen alpha-1(XI) chain
A0A0G2KAN1	Collagen alpha-2(1) chain
M0RBJ7	Complement C3
Q6IN11	Connective tissue growth factor
P14841	Cystatin-C
G3V6X1	Fibulin 2
Q9WVH8	Fibulin 5
C0JPT7	Filamin A
Q62632	Follistatin-related protein 1
Q64599	Hemiferrin
P12843	Insulin-like growth factor-binding protein 2
F1LNH3	Collagen alpha-2(IV) chain
F1LQ00	Collagen alpha-2(V) chain
D3ZUL3	Collagen alpha-1(VI) chain
D3ZFH5	Inter-alpha-trypsin inhibitor heavy chain 2
O35806	Latent-transforming growth factor beta-binding protein 2
Q5M7T5	Serine (Or cysteine) peptidase inhibitor, clade C (Antithrombin), member 1
P16975	SPARC
Q71SA3	Thrombospondin 1
P85972	Vinculin

Table 5-1 List of protein commonly produced by the three CM samples

5.4.1 Extracellular region proteins

Based on Panther Classification Systems (PCS) (http://www.pantherdb.org), proteins detected from the samples can be divided based on their cellular components which are cell junction, cell part, extracellular matrix, extracellular region, macromolecular complex, membrane, organelle and synapse. Our interest in proteomic analysis were in proteins of extracellular region as these proteins probably produced by the cells and not part of the cells.

For extracellular region proteins, we detected 19 from BMSCs; 12 from PDLSCs and 17 from DPSCs as presented in Figure 5.5.

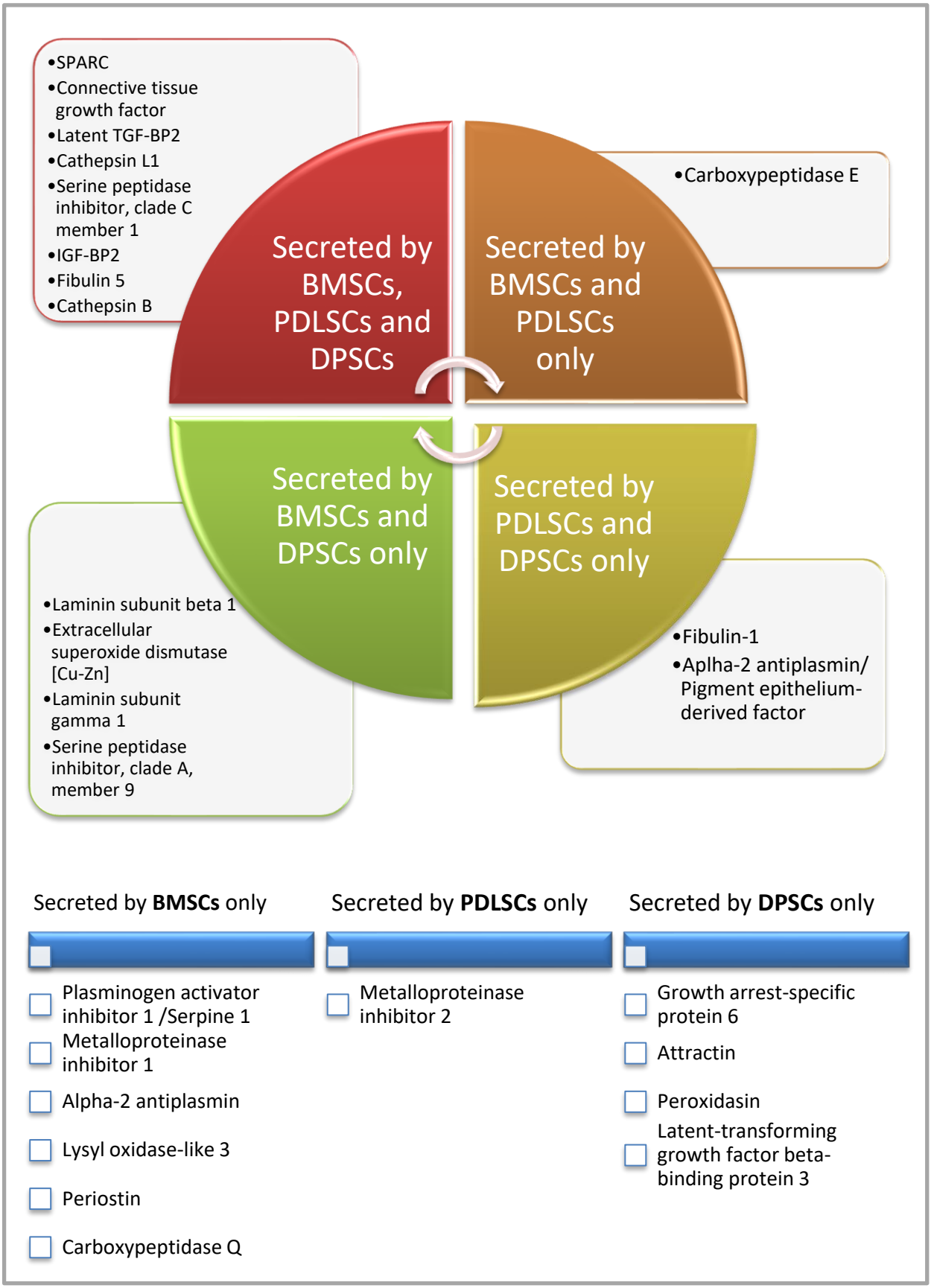


Figure 5.5 List of exracellular region proteins detected from BMSCs, PDLSCs and DPSCs samples.

5.4.2 TMT protein quantification of the normoxia and hypoxia samples

Quantitative proteomic profiles were analysed using more CM samples from BMSCs, PDLSCs and DPSCs (both normoxia and hypoxia samples of each cell-type), giving rise to 9 channels, and one master sample, containing equal protein quantitiy of all samples, and labelled with Tandem Mass Tag (TMT) and pooled to one sample. The pooled samples were run through LC-MS/MS in two technical replicates and the peptides were identified from the MS/MS Spectra using both Sequest and Mascot protein searches. The abundance of the identified proteins was normalised to the master sample (Appendix 3). Total of 22 proteins were detected but 11 proteins were labelled across the samples and quantified.

5.4.2.1 Principal Component Analysia (PCA) of the secreted proteins

The average value of each proteins were analysed via Clustvis (Metsalu and Vilo 2015) in order to visualize the clustering of the protein samples to each cell-types. The value of Day 2 samples was included in order to assess whether the Day 2 and Day 3 samples behaved in similar manner.

From the PCA plot, we could observe that every cell-types formed distinctive clusters as shown in Figure 5.6. The cluster by the BMSCs is more widespread especially between normoxia and hypoxia samples in comparison to PDLSCs and DPSCs. In PDLSCs cluster, the difference between normoxia and hypoxia samples was almost not present.

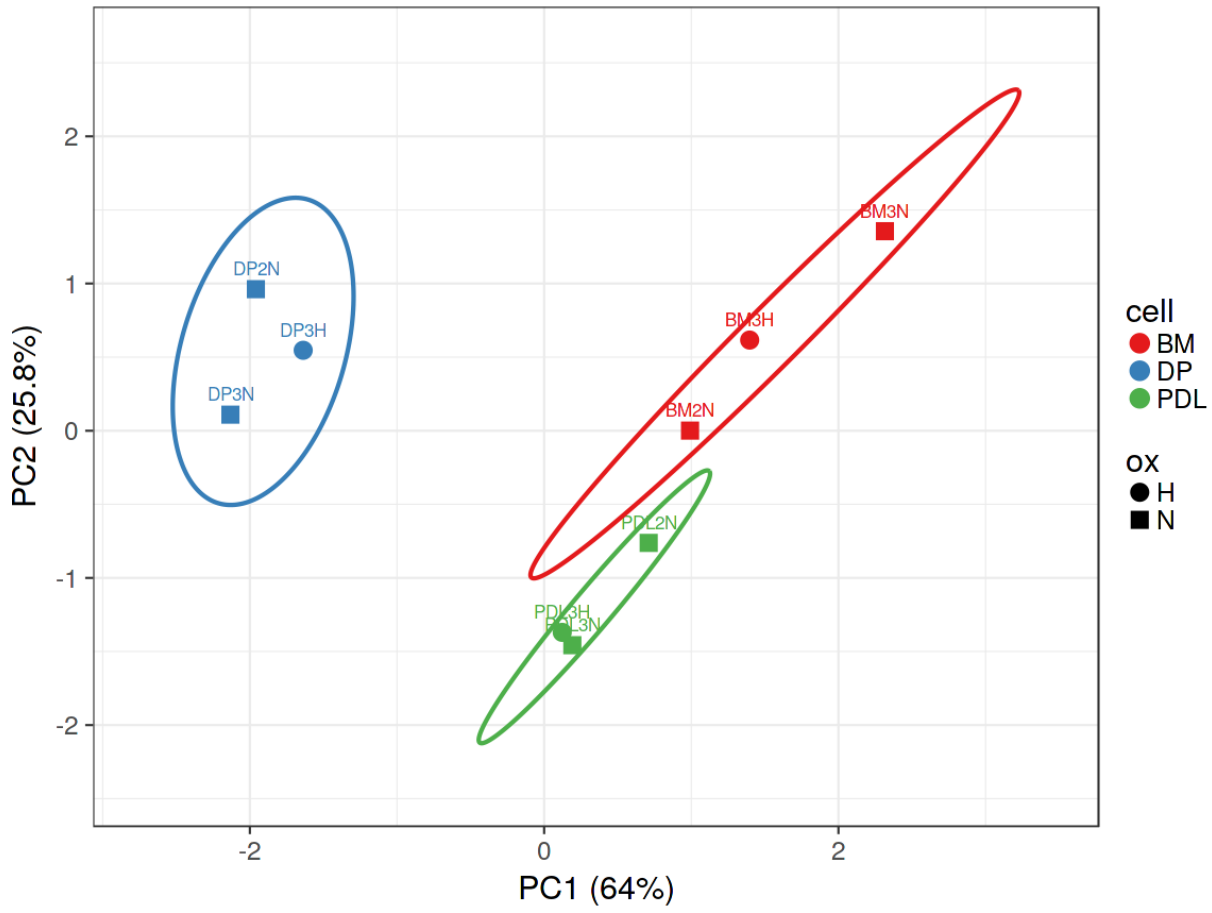


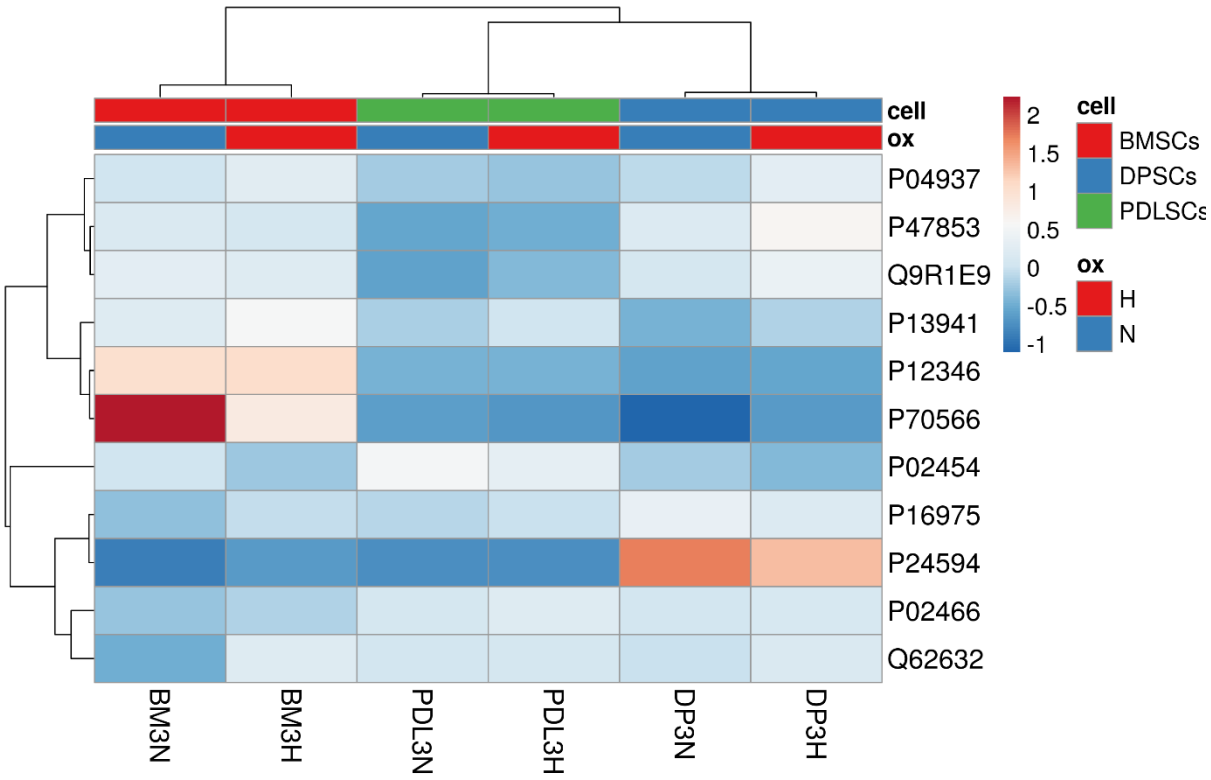
Figure 5.6 PCA plot of protein clusters of BMSCs, PDLSCs and DPSCs in different oxygen incubations.

No scaling is applied to the protein samples as the value has been scaled to the ratio of master sample. Singular Value Decomposition (SVD) with imputation was used as the method to calculate the principal components. X and Y axis show principal component 1 and principal component 2 that explain 64% and 25.8% of the total variance, respectively. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. N = 9 data points.

BM 3N: CM of BMSC from normoxia incubation and collected on Day 3
BM 3H: CM of BMSC from hypoxia incubation and collected on Day 3
BM 2N: CM of BMSC from normoxia incubation and collected on Day 2
PDL 3N: CM of PDLSC from normoxia incubation and collected on Day 3
PDL 3H: CM of PDLSC from hypoxia incubation and collected on Day 3
PDL 2N: CM of PDLSC from normoxia incubation and collected on Day 2
DP 3N: CM of DPSC from normoxia incubation and collected on Day 3
DP 3H: CM of DPSC from hypoxia incubation and collected on Day 3
DP 2N: CM of DPSC from normoxia incubation and collected on Day 2

5.4.2.2 Heatmap

Data from the day 3 samples were further analysed with Heatmap which is the data analysis tool that uses the color-coded graphics system based on the samples and its quantity. Based on the analysis as presented in Figure 5.7, heatmap suggested that there were three clusters of protein which define the three cell types. First group is proteins of BMSCs which are Serotransferrin (P12346) and Tropomodulin-2 (P70566). Second group of protein identified is for DPSCs, which is the Insulin-like of growth factor binding protein 5 (P24594). Collagen alpha-(1) chain is the only protein that defined PDLSCs (P02454).



Accession Number	Protein name
P04937	Fibronectin
P47853	Biglycan
Q9R1E9	Connective tissue growth factor
P13941	Collagen alpha-1(III) chain
P12346	Serotransferrin
P70566	Tropomodulin-2
P02454	Collagen alpha-1(I) chain
P16975	SPARC
P24594	Insulin-like growth factor-binding protein 5
P02466	Collagen alpha-2(I) chain
Q62632	Follistatin-related protein 1

Figure 5.7 Heatmap of the proteins detected in six CM samples

Rows are centered; no scaling is applied to rows. Nipals PCA is used for missing value estimation. Both rows and columns are clustered using correlation distance and average linkage. 11 rows, 6 columns.

5.4.2.3 Relative Changes to Protein Secretion between different cell types

From the list of protein detected through TMT Mass spectrometry, we highlighted extracellular region proteins. There were three extracellular region proteins identified in BMSCs (SPARC, Connective Tissue Growth Factor and Fibronectin) and additional one protein secreted by PDLSCs and DPSCs (Insulin-like Growth Factor Binding Protein 5). Other proteins identified was either proteins of cell parts, macromolecular complex, organelle or membrane in origin.

The first part of data analysis was to evaluate the relative changes in protein secretion between two types of dental MSCs compared to BMSCs as control sample. The normalised value of proteins secreted by the PDLSCs and DPSCs was divided with the value of BMSCs that served as control cells in this experiment. The ratio was logged to 2(fold) and any value of >1 was considered increase secretion to the protein and <-1 considered as reduction in protein secretion.

The data showed that DPSCs produced relatively higher SPARC than BMSCs and both PDLSC and DPSCs produced relatively higher Follistatin-related protein-1 but relatively lower serotransferrin, tropomodulin-2 and connective tissue growth factor than BMSCs (Table 5-2).

Accession	Protein Name	MW (kDa)	Relative ratio PDLSCs/BMSCs	Log2 (fold)	Findings	Relative ratio DPSCs/BMSCs	Log2 (fold)	Findings
P02454	Collagen alpha-1(I) chain	137.9	1.46978	0.5556	No changes	0.796245	-0.32871	No changes
P02466	Collagen alpha-2(I) chain	129.5	1.671053	0.740757	No changes	1.604441	0.682071	No changes
P04937	Fibronectin	272.3	0.779643	-0.35911	No changes	0.903463	-0.14646	No changes
P12346	Serotransferrin	76.3	0.369076	-1.43801	-	0.307464	-1.70151	-
P13941	Collagen alpha-1(III) chain	138.9	0.74168	-0.43113	No changes	0.559429	-0.83797	No changes
P16975	SPARC	34.3	1.27782	0.353684	No changes	2.034065	1.024366	+
P47853	Biglycan	41.7	0.406841	-1.29746	-	1.030603	0.043489	No changes
P70566	Tropomodulin-2	39.5	0.197933	-2.33692	-	0.065289	-3.93703	-
Q62632	Follistatin-related protein 1	34.6	2.467192	1.30287	+	2.312336	1.209351	+
Q9R1E9	Connective tissue growth factor	37.7	0.359143	-1.47737	-	0.850082	-0.23433	-

Table 5-2 Relative changes to proteins identified in PDLSCs and DPSCs samples compared to BMSCs

5.4.2.4 Relative Changes to Protein Content between different day of CM collection

Further analysis involved the data from different days of CM collection the procedure in 1.1.1.1 were repeated for data between day 3 and day 2. Most of the proteins detected showed no changes between Day 3 and Day 2; except for Tropomodulin-2 which was upregulated in Day 3 CM and Folistatin-related protein 1 which was reduced in Day 3 CM (Table 5-3).

Serotransferrin is the only protein that showed reduction in Day 3 CM of PDLSCs, as the Tropomodulin for the DPSCs; whereas the other detected proteins did not show any changes between Day 2 and Day 3 CM (Table 5-4 and Table 5-5).

BMSCs					
Accession	Protein Name	MW (kDa)	Relative ratio Day 3/ Day 2	Log2 (fold)	Findings
P02454	Collagen alpha-1(I) chain	137.9	1.336597	0.418565	No changes
P02466	Collagen alpha-2(I) chain	129.5	0.502064	-0.99406	No changes
P04937	Fibronectin	272.3	0.69868	-0.5173	No changes
P12346	Serotransferrin	76.3	1.141312	0.190693	No changes
P13941	Collagen alpha-1(III) chain	138.9	1.935583	0.952768	No changes
P16975	SPARC	34.3	0.796743	-0.32781	No changes
P47853	Biglycan	41.7	1.216867	0.283172	No changes
P70566	Tropomodulin-2	39.5	2.110142	1.07734	+
Q62632	Follistatin-related protein 1	34.6	0.317765	-1.65397	-
Q9R1E9	Connective tissue growth factor	37.7	1.246407	0.317775	No changes

Table 5-3 Relative changes to proteins identified in BMSCs samples between Day 3 and Day 2 of CM collection
(+) Increased amount of protein secretion; (-) Decreased amount of protein secretion.

PDLSCs					
Accession	Protein Name	MW (kDa)	Relative ratio Day3/Day2	Log2 (fold)	Findings
P02454	Collagen alpha-1(I) chain	137.9	1.662351	0.733225	No changes
P02466	Collagen alpha-2(I) chain	129.5	0.958491	-0.06116	No changes
P04937	Fibronectin	272.3	0.762051	-0.39204	No changes
P12346	Serotransferrin	76.3	0.396563	-1.33438	**-**
P24594	Insulin-like growth factor-binding protein 5	30.3	0.880527	-0.18356	No changes
P13941	Collagen alpha-1(III) chain	138.9	0.76588	-0.38481	No changes
P16975	SPARC	34.3	1.695946	0.76209	No changes
P47853	Biglycan	41.7	0.747107	-0.42061	No changes
P70566	Tropomodulin-2	39.5	0.844232	-0.24429	No changes
Q62632	Follistatin-related protein 1	34.6	0.659187	-0.60124	No changes
Q9R1E9	Connective tissue growth factor	37.7	0.519666	-0.94434	No changes

Table 5-4 Relative changes to proteins identified in PDLSCs samples between Day 3 and Day 2 CM collection

(+) Increased amount of protein secretion; (-) Decreased amount of protein secretion.

DPSCs					
Accession	Protein Name	MW (kDa)	Relative ratio Day 3/Day 2	Log2 (fold)	Findings
P02454	Collagen alpha-1(I) chain	137.9	1.308503	0.387917	No changes
P02466	Collagen alpha-2(I) chain	129.5	0.906599	-0.14146	No changes
P04937	Fibronectin	272.3	0.732143	-0.4498	No changes
P12346	Serotransferrin	76.3	0.990458	-0.01383	No changes
P24594	Insulin-like growth factor-binding protein 5	30.3	0.801362	-0.31947	No changes
P13941	Collagen alpha-1(III) chain	138.9	1.146331	0.197024	No changes
P16975	SPARC	34.3	0.994346	-0.00818	No changes
P47853	Biglycan	41.7	0.669982	-0.5778	No changes
P70566	Tropomodulin-2	39.5	0.30738	-1.7019	**-**
Q62632	Follistatin-related protein 1	34.6	0.937234	-0.09352	No changes
Q9R1E9	Connective tissue growth factor	37.7	0.667961	-0.58216	No changes

Table 5-5 Relative changes to protein identified in DPSCs samples between Day 3 and Day 2 of CM collection

(+) Increased amount of protein secretion; (-) Decreased amount of protein secretion.

5.4.2.5 Relative Changes to Protein Secretion between normoxia and hypoxia incubations

Further analysis involved the normoxia and hypoxia data. The only protein deemed to be highly secreted in hypoxia incubation is Follistatin-related protein 1 by the BMSCs and Tropomodulin-2 by the DPSCs. There were no changes in proteins identified from PDLSCs normoxia and hypoxia samples.

BMSCs					
Accession	Protein Name	MW (kDa)	Relative ratio Hypoxia/Normoxia	Log2 (fold)	Effect of hypoxia incubation to the protein secretion
P02454	Collagen alpha-1(I) chain	137.9	0.768315	-0.38023	No changes
P02466	Collagen alpha-2(I) chain	129.5	1.266447	0.340787	No changes
P04937	Fibronectin	272.3	1.218258	0.28482	No changes
P12346	Serotransferrin	76.3	1.013626	0.019525	No changes
P13941	Collagen alpha-1(III) chain	138.9	1.246434	0.317807	No changes
P16975	SPARC	34.3	1.387585	0.472576	No changes
P47853	Biglycan	41.7	0.951395	-0.07188	No changes
P70566	Tropomodulin-2	39.5	0.606546	-0.72131	No changes
Q62632	Follistatin-related protein 1	34.6	2.952756	1.562062	++
Q9R1E9	Connective tissue growth factor	37.7	0.954695	-0.06689	No changes

Table 5-6 The relative changes to protein secretion by BMSCs in hypoxia incubation compared to normoxia incubation
(++) Increased amount of protein secretion.

PDLSCs					
Accession	Protein Name	MW (kDa)	Relative ratio Hypoxia/Normoxia	Log2 (fold)	Effect of hypoxia incubation to the protein secretion
P02454	Collagen alpha-1(I) chain	137.9	0.888	-0.17212	No changes
P02466	Collagen alpha-2(I) chain	129.5	1.126	0.171817	No changes
P04937	Fibronectin	272.3	0.917	-0.12571	No changes
P12346	Serotransferrin	76.3	0.981	-0.02806	No changes
P24594	Insulin-like growth factor-binding protein 5	30.3	1.182	0.240771	No changes
P13941	Collagen alpha-1(III) chain	138.9	1.123	0.167644	No changes
P16975	SPARC	34.3	1.032	0.045265	No changes
P47853	Biglycan	41.7	1.095	0.131106	No changes
P70566	Tropomodulin-2	39.5	0.922	-0.11768	No changes
Q62632	Follistatin-related protein 1	34.6	1.061	0.084933	No changes
Q9R1E9	Connective tissue growth factor	37.7	1.450	0.535596	No changes

Table 5-7 Relative changes to protein secretion by PDLSCs in hypoxia incubation compared to normoxia incubation

DPSCs					
Accession	Protein Name	MW (kDa)	Relative ratio Hypoxia/Normoxia	Log2 (fold)	Effect of hypoxia incubation to the protein secretion
P02454	Collagen alpha-1(I) chain	137.9	0.819436	-0.2873	No changes
P02466	Collagen alpha-2(I) chain	129.5	1.093798	0.129346	No changes
P04937	Fibronectin	272.3	1.384437	0.469299	No changes
P12346	Serotransferrin	76.3	1.044316	0.062558	No changes
P24594	Insulin-like growth factor-binding protein 5	30.3	1.36119	0.444868	No changes
P13941	Collagen alpha-1(III) chain	138.9	0.863044	-0.21249	No changes
P16975	SPARC	34.3	0.844073	-0.24456	No changes
P47853	Biglycan	41.7	1.356332	0.43971	No changes
P70566	Tropomodulin-2	39.5	2.955145	1.563229	++
Q62632	Follistatin-related protein 1	34.6	1.191827	0.253175	No changes
Q9R1E9	Connective tissue growth factor	37.7	1.246124	0.317448	No changes

Table 5-8 Relative changes to protein secretion by DPSCs in hypoxia incubation compared to normoxia incubation

(++) Increased amount of protein secretion.

5.5 Discussion

5.5.1 Variation in secretomes

Since the research on secretomes has begun there has been a wide variation in the methods for collection of the conditioned media used to study the secretome from cultured cells. The differences in the incubation types, addition of any pharmacologic agents in the culture media, duration of the culture before CM collection, filtration of the CM and many more approaches, which have caused variation in the research findings especially involving the content of certain growth factors and other biological mediators.

One of the noteworthy differences found with secretomes was based on when the CM was collected. Most of studies collected the CM at 48 hours (Osugi, Katagiri et al. 2012, Inukai, Katagiri et al. 2013, Yalvaç, Yarat et al. 2013, Ando, Matsubara et al. 2014, Fujio, Xing et al. 2015, Ogata, Katagiri et al. 2015, Park, Bae et al. 2015). There were only limited studies that collected the CM on the third day of culture or longer. The reason for collecting CM at longer periods than 2 days is to obtain a richer content of growth factors or bioactive molecules, however the media used would be deprived due to the cells metabolic activity (Paschalidis, Bakopoulou et al. 2014). One study collected the CMs of human umbilical cord stem cells for three days to test in *in vivo* experiments. Although the CM was a product of protein accumulated longer than 48 hours, the CM still provided a positive effect on bone regeneration *in vitro* and *in vivo* (Wang, Xu et al. 2015). Another study collected the CMs of rat DPSCs, BMSCs and ADSCs after 3 days for mass spectrometry analysis and showed various proteomic profiles that linked the MSC secretome with angiogenesis, cell migration and inflammatory response (Tachida, Sakurai et al. 2015).

Longer than 3 days collection of the CM was reported in another study in which the researchers collected the CM from DPSC cultures on every 4th day up to 24 days however the culture media contained 0.5% FBS. The study found that the cell viability via MTT assay was higher in earlier collection periods (Paschalidis, Bakopoulou et al. 2014). Thus, in this study, CM was collected on

the second and third day but at less than 4 days to reduce the chances of contamination into the CM with byproducts of the cell metabolic activity. Data showed that the longer culture time allowed more growth factors to accumulate in the CM.

Another variability in CM processing technique that should be highlighted is the filtration of the CMs. Only a few research groups have reported that their CMs were filtered (Kim, Choi et al. 2013, Yalvaç, Yarat et al. 2013, Paschalidis, Bakopoulou et al. 2014, Tachida, Sakurai et al. 2015, Kumar, Kumar et al. 2017, Kumar, Kumar et al. 2018). The study presented here suggested that a 0.2μm filter membrane entrapped and concentrated growth factors such as VEGF, TGF-β1 and IGF-1 as the filtrated samples showed significantly less growth factors compared with the unfiltrated samples. This study also found that the level of TGF-β1 in DPSCs samples was relatively low although this may be because the CM samples for this particular experiment were collected from the second day of the experiment (Figure 5.1).

5.5.2 Protein identification via mass spectrometry

Proteomic studies of the proteins secreted by the MSCs have been reported in many studies and this has resulted in merely protein inventories and catalogues of their contents (Celebi, Elcin et al. 2010, Ma, Cui et al. 2014) or the proteins secreted by the cells which is known as the secretome (Tachida, Sakurai et al. 2015).

Data presented here focused on the proteins secreted by the cells under the category of extracellular region proteins based on the PANTHER (Protein annotation through evolutionary relationship) Classification System (Thomas, Campbell et al. 2003). The system utilised an organized database of all proteins and gene sequences available and consists of 82 complete genomes and classifies the genes and proteins based on their functions and cellular locations (Mi, Muruganujan et al. 2013). In this study, the main extracellular proteins found in all three MSC CMs samples were SPARC, connective tissue growth factor, TGF-β2, and IGF-BP2.

SPARC or osteonectin is a type of glycoprotein that can be found in connective tissues such as bone, periodontal ligaments and dental pulp. The function of this glycoprotein is to bind calcium and collagen (Maurer, Hohenadl et al. 1995). It is a collagen binding protein which plays an important role in collagen fibril synthesis in the extracellular matrix and SPARC deletion subsequently reduces collagen in PDL (Trombetta, Bradshaw et al. 2010). In dental pulp, SPARC is reported to be secreted by DPSC and odontoblasts and involved in pulpal repair processes (Reichert, Störkel et al. 1992). This is likely due to the effect of SPARC in increasing dental pulp cell metabolic activity (Shiba, Uchida et al. 2001) and cell migration (Pavasant and Yongchaitrakul 2008) in a few *in vitro* studies involving human dental pulp cells.

Interestingly, relative quantification using mass spectrometry analysis showed that there was increased secretion of osteonection/SPARC by the DPSC compared with BMSC. This is in contrast with a recent study by Tachida an coworkers that showed no differences in their relative quantification using mass spectrometry analysis (Tachida, Sakurai et al. 2015). This study showed no changes in the SPARC secretion by PDLSC compare with BMSC. Another study that compared SCAPs and BMSCs also showed increased relative amounts of secreted SPARC by SCAPs (Shi, Yuming et al. 2016). Apart from Tachida (2015) and Shi (2016), there is general lack of research in proteomic quantification involving dental stem cell secretomes.

CTGF is growth factor that is known to induce differentiation of stem cells towards the fibroblast phenotype and production of type I collagen (Lee, Moioli et al. 2006), Notably is was reported to be up-regulated in early osteoblast differentiation (Luo, Kang et al. 2004). The current study found that CTGF was secreted at lower levels by PDLSCs and DPSCs compared with BMSCs. This finding is similar to the one reported by Tachida and coworkers who showed a higher CTGF secretion by BMSCs compared with DPSCs (Tachida, Sakurai et al. 2015). Currently no other secretome study has reported on the level of CTGF in any dental mesenchymal stem cells.

From the result, the PCA plot able to show distinct clusters of proteins for each of the three cell types. Among these three cell types, the proteins secreted by PDLSCs under both normoxia and

hypoxia incubations exhibited a similar profile. This is in line with the analysis from the heatmap generated which indicated no distinct proteins secreted for PDLSCs compared with the BMSCs and DPSCs protein samples (Figure 5.7 and Table 5-7).

All proteins listed showed secretion both in normoxia and hypoxia incubations. Based on the heatmap analysis, tropomodulin-2 was the only protein shown to be strongly associated with BMSCs, but the production was lower under hypoxia incubation. IGF-BP5 was the only protein associated with DPSCs which was reduced under hypoxia incubation compared with normoxia incubation.

The proteomic data from the Tandem Mass Tag analysis can be considered preliminary. No other study has compared the proteomic profiles between PDLSCs, DPSCs and BMSCs from the same species. However, one study reported relative quantification of the proteins detected in the rat BMSCs, DPSCs and ADSCs (Tachida, Sakurai et al. 2015). Less than 15 proteins were detected, and the relative quantification was performed based on the abundance value of each proteins in comparison with the protein detected in the master sample. This number of proteins detected was smaller compared to Tachida's work.

The comparative analysis of day 2 and day 3 CMs showed that Folistatin related protein-1 was the only protein detected in these experiments to be reduced in day 3 CM of BMSCs samples. However, reduced oxygen incubation increased levels of this protein. Follistatin related protein-1 is an activin binding protein and interestingly activin belongs to the TGF-β1 superfamily and is involved in stem cell differentiation (Pauklin and Vallier 2015). However, the exact role of folistatin related protein-1 in BMSCs, has not yet been clarified. There remains the possibility that this protein was degraded in the medium from relatively long duration cultures.

Conversely, Tropomodulin-2 was increased in day 3 CM compared with day 2 BMSC CM. This protein regulates actin which is one of the important cellular cytoskeletal proteins that also plays an important role in cell growth, signalling and differentiation (Yourek, Hussain et al. 2007). However,

tropomodulin-2 was reduced in day 3 CM of the DPSCs samples which may be due to degradation of the protein in day 3 CM.

Another protein that appears to be increased in day 3 CM is Serotransferrin. This iron-binding protein was higher in day 3 PDLSC CM compared with day 2 CM. It may be speculated that this is one of the proteins prone to degradation in longer term culture medium. In fact, this protein was not detected in Tachida and coworkers proteomic data (Tachida, Sakurai et al. 2015).

The proteomic data presented in this study showed that the VEGF and TGF-B1 were not detected in the mass spectrometry analysis of the samples. This is probably because the technique used was shot-gun approach which has limited sensitivity. This could be overcome by using different techniques of mass spectrometry analysis such as in Selected Reaction Monitoring (SRM) in which the technique able to detect and select any particular ions for example the VEGF or TGF-β and its fragments in the samples. This technique was reported to be more reliable in detecting low abundance protein especially in complex mixture of protein samples (Kupcova Skalnikova 2013). Other than that, the presence of highly abundant proteins such as albumin could mask the detection of low abundance VEGF and TGF-β1 (Kupcova Skalnikova 2013). Thus, in order to overcome this problem, total elimination of albumin in the CM samples should be done prior to mass spectrometry analysis by treating the samples with anti-albumin.

5.5.3 Conclusion

Based on the results presented in this chapter, it can be concluded that different collection days, different oxygen concentrations and the filtration of the CMs alters the protein content of secretomes. Indeed, a list of proteins detected in the CM from cultures of three sources of MSCs by mass spectrometry proteomic analysis was presented indicating a complex and diverse mixture of proteins.

CHAPTER 6

RESULT 4

Comparative Analysis of The Bioeffects of MSC Secretomes

6 RESULT 4

6.1 Introduction

The focus of this chapter is to analyse the effects of the collected secretome from BMSCs, PDLSCs and DPSCs on the pre-osteoblast cell line, MC3T3. The objective of this chapter is:

i. To assess the effect of the CM on pre-osteoblast cells proliferation.
ii. To evaluate the potential of the CM in inducing differentiation and mineralisation in pre-osteoblast cell cultures.
iii. To evaluate the effect of the CM on pre-osteoblasts cell migration.

This study assessed the effect of the CM on MC3T3 cell proliferation using MTT Assay. The cells were cultured with the respective CM for three days in normal oxygen concentration. This study also evaluated the potential of the CM in inducing the differentiation of the MC3T3 through Alkaline Phosphatase Assay (ALP Assay) as ALP is one of the markers for osteogenic differentiation. The last part of this chapter is to evaluate the effect of the CM on cell migration and chemotaxis.

6.2 The effect of concentrated CM on pre-osteoblast viability

To evaluate the effect of the CM from the different cell types and incubation type, the pre-osteoblast cells (MC3T3) were cultured in serum free media with addition of six different groups of culture media. The first three groups were cultured in Day 3 CM of BMSCs (BMSC N), PDLSCs (PDLSC N) and DPSCs (DPSC N) of normoxia incubation. Another three groups were from each cell type cultured in hypoxia (labelled as BMSC H, PDLSC H and DPSC H). All CM used in this experiment were from third day collection (Day-3 CM).

The culture media were adjusted to contain 100µg/ml of protein of the concentrated CM (calculated from the BCA assay mentioned in section 2.9). The cells were cultured for three days and MTT assay was performed on each day to evaluate the number of viable cells in comparison to cells cultured with 10% FBS or serum free media only. The results of as presented in Figure 6.1 showed that MTT absorbances all the test groups were significantly higher than the SFM controls. Indeed, MTT values plateaud at Day 1 which is markedly different from the positive control group which showed increasing absorbance in the second and third day of culture. In fact, there was a reduction of the MTT staining on the third day of culture in each test group.

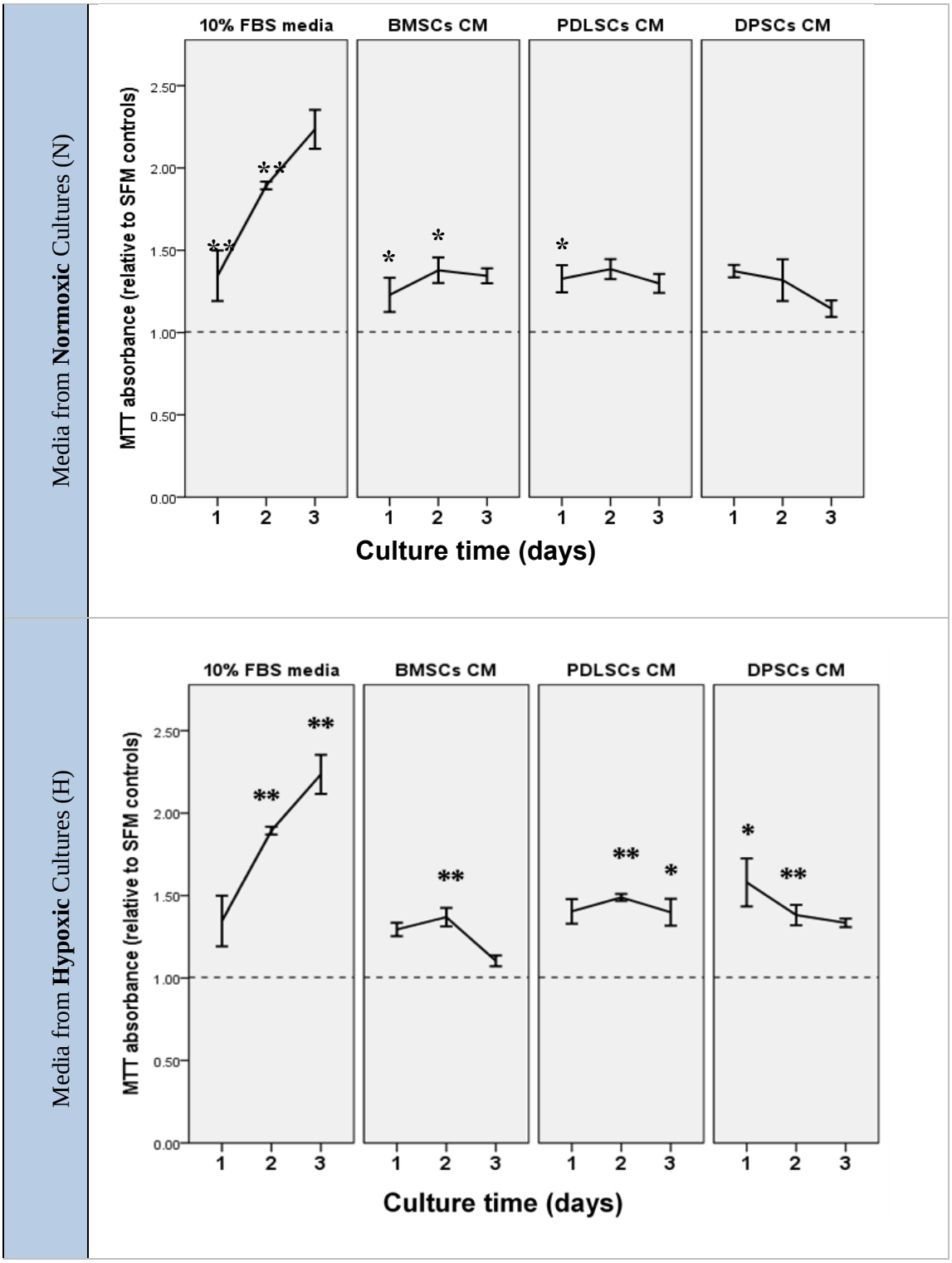


Figure 6.1 Viable cell numbers between three days in different collected CM

Statistical comparison was performed between each time point with the control negative (SFM) with *p -value<0.05 and **p value<0.001; n=5 (technical replicates). Reference line indicates the average MTT absorbance of control negative group.

6.2.1 The effect of collection Day of the CM on viability of MC3T3

The evaluation of the viable cell number was further compared between CM collected on second day of culture (2N or 2H) with CM from three days of culture (3N or 3H) in order to observe the effect of Day 2 and Day 3 CM. In this experiment, the MC3T3 cells were cultured as the experiment in section 6.2 for 2 days.

The results showed that almost all groups produced higher average MTT absorbance compared to the control negative group except for the group cultured in Day 2 CM of BMSC under normoxia incubation. However, as shown in Figure 6.2 all CM cultured for three days produce a more favourable culture environment for the MC3T3 cells compared to control negative group, Even the Day 3 CM showed favourable cell viability in almost every groups compared to the Day 2 CM. In fact, the difference between Day 2 and Day 3 groups of BMSC CM and PDLSC CM in normoxia incubation was statistically significant. Although significant difference was not observed for CM of DPSC in normoxia incubation, the Day 3 CM of DPSC showed higher average absorbance compared to Day 2 CM indicating increased number of viable cells.

For the CM derived from hypoxia incubation, the significant difference was still observed between Day 3 CM and Day 2 CM from BMSCs. Furthermore, the average absorbance values for both Day 2 and Day 3 CMs were higher compared with the normoxic groups. As Day 3 CM showed enhanced cell viability especially when the cells were cultured in normoxia incubation, Day 3 CMs were used in subsequent experiments.

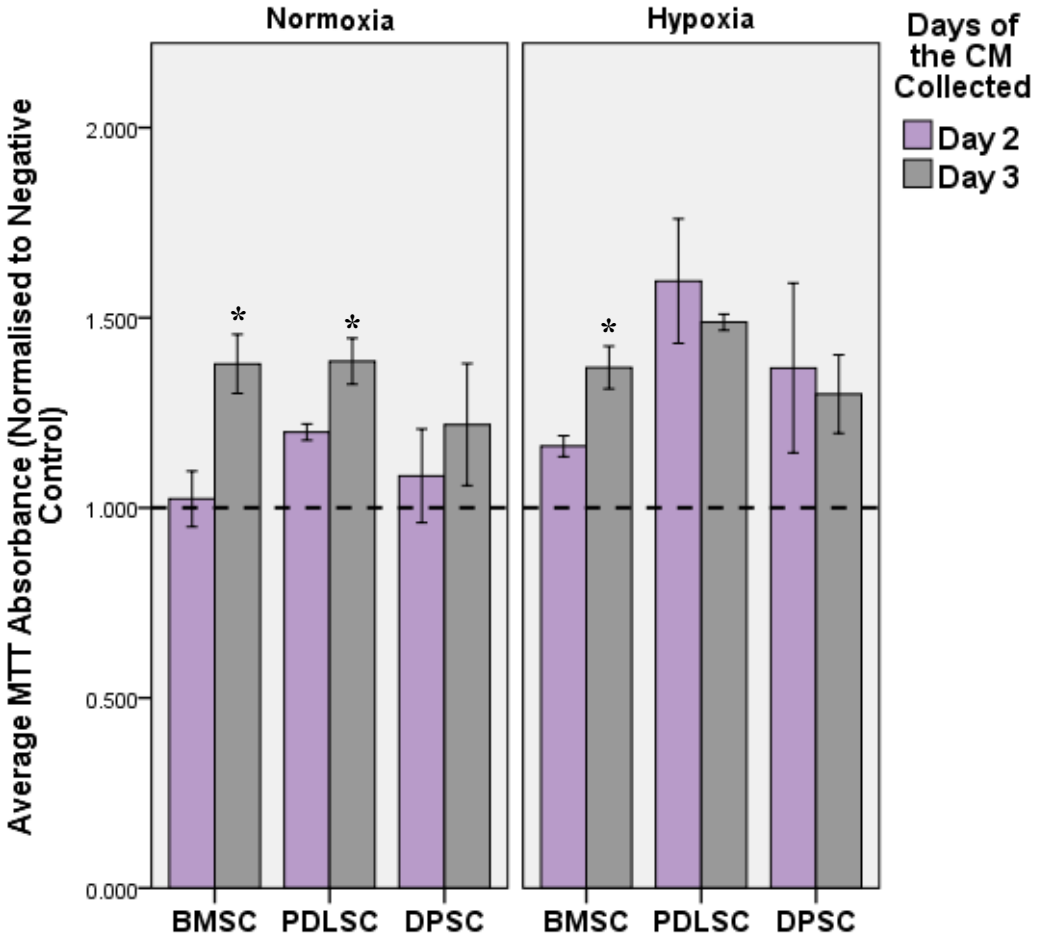


Figure 6.2 Effect of different in days of media collection to the viability of Mc3T3 Statistical comparison was performed between Day 2 and Day 3 of CM collection with *p -value<0.05. n=6 (technical replicates). Reference line indicates the average MTT absorbance of control negative group

6.2.2 The effect of different protein concentrations on viability of MC3T3

Further experiment as in section 6.2.1 was performed to test the effects of different protein concentrations in the CM samples (100µg/ml vs 200µg/ml). The MC3T3 were cultured with Day 2-CM and the MTT assay was performed after 2 days.

The result showed that there was a modest increase in cell numbers with increased protein concentration with significant difference reached for CM from PDLSCs of hypoxia incubation (p-value= 0.031) (Figure 6.3).

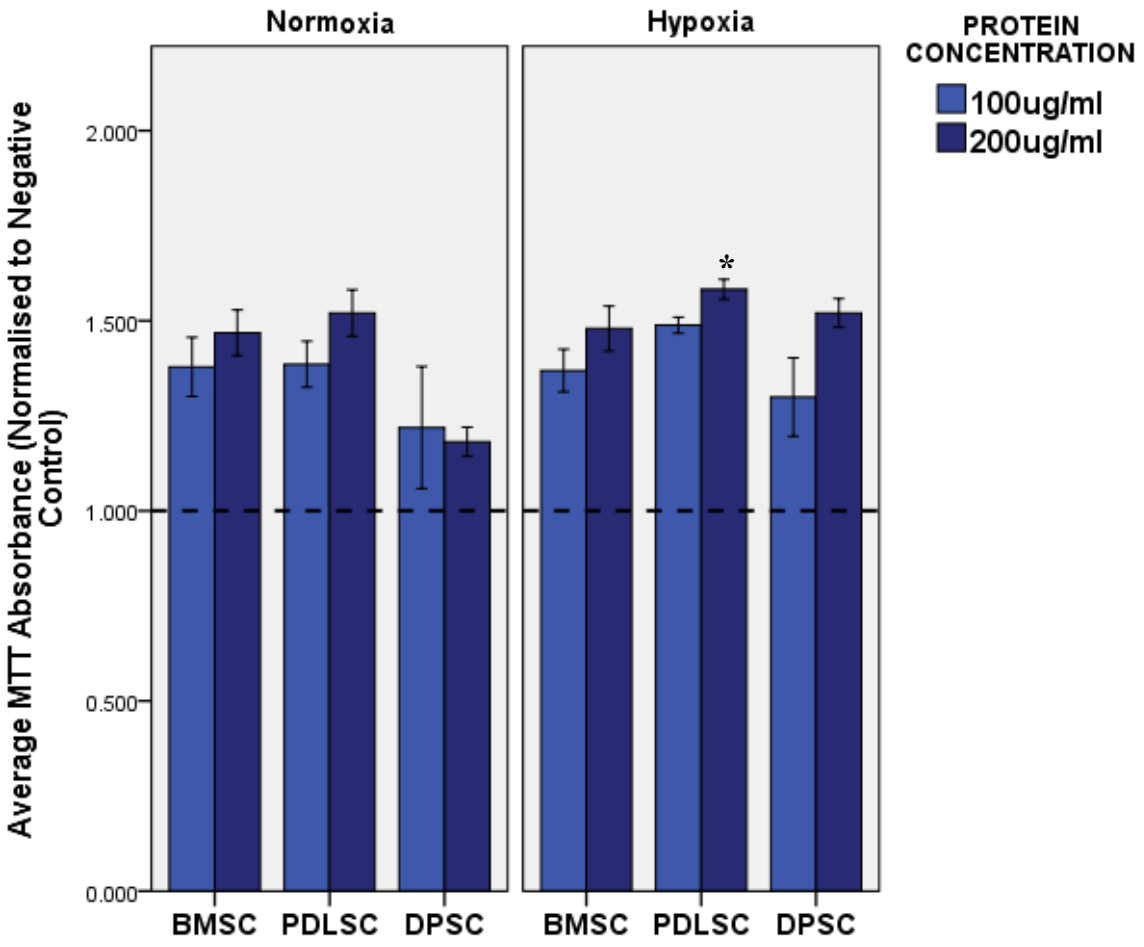


Figure 6.3 Effect of protein concentration of the CM to the viability of Mc3T3
Statistical comparison was performed between 100ug/ml and 200ug/ml of the protein concentration with *p -value<0.05. n=6 (technical replicates). Reference line indicates the average MTT absorbance of control negative group.

6.3 The effect of concentrated CM on ALP activity of pre-osteoblast cells

The effect of the CM on the ALP activity of the MC3T3 pre-osteoblast cells was tested. Prior to that, the commercially available ALP Assay kit (SIGMA*FAST*™) was used to analyse SAOS2 cells which are known to posses high levels of AlP activity (Rodan, Imai et al. 1987).

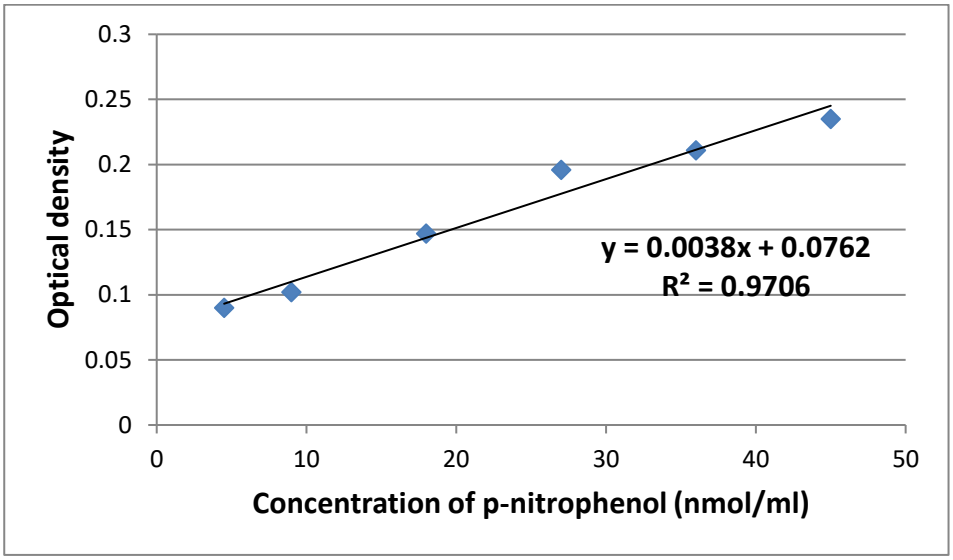


Figure 6.4 Standard Curve for ALP Assay

Based on the above formula, we calculated the p-NP concentration produced by the assay and presented in Figure 6.5. The level of p-NP was significantly higher when the SAOS2 cells cultured in osteogenic media which contained 10% FBS compared to when the cells were cultured in serum free media which serves as control group.

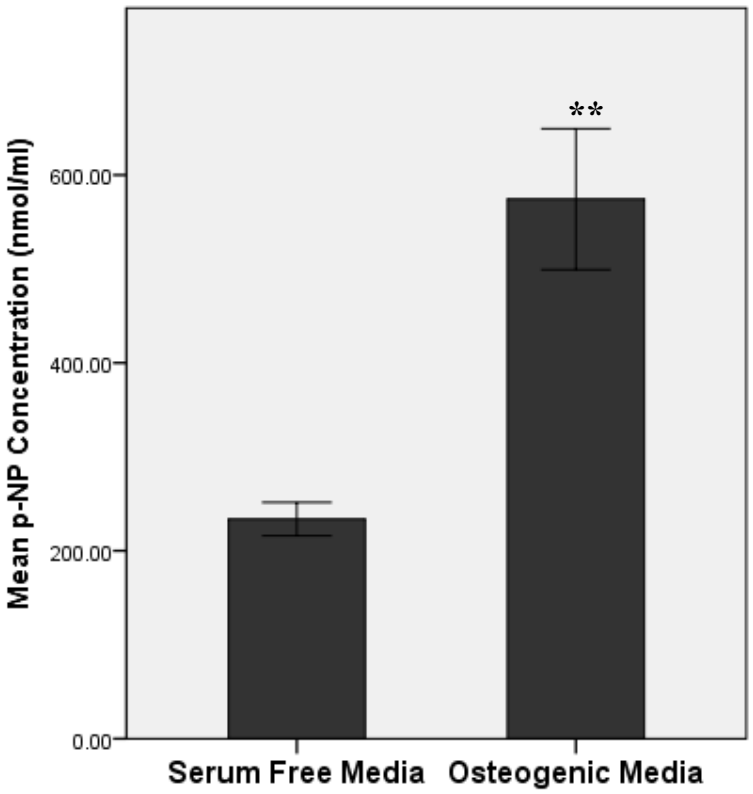


Figure 6.5 Level of the p-NP produced by the SAOS2 cells in ALP Assay

The ALP activity of the SAOS2 cells cultured with osteogenic media was compared with control group (10% FBS primary culture media). The statistical analysis showed significant difference with **p-value=0.001. N=6 (technical replicates).

As the SIGMA*FAST*™ ALP Assay kit was now shown to reliable measure different levels of ALP activities, we then proceeded to use this kit on MC3T3 cells .

The result showed that the ALP activity of MC3T3 cells was relatively low in control cultures (underlyng their undifferentiated state) but was significantly increased in osteogenic media suggesting stimulation of osteogenic differentiation (Figure 6.6).

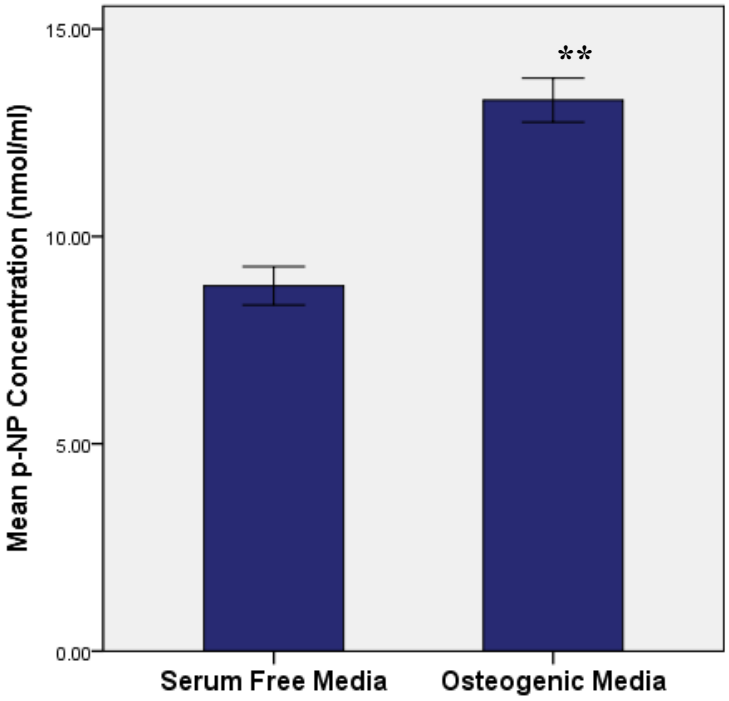


Figure 6.6 Level of p-NP produced by MC3T3 cells in ALP Assay
The ALP activity of the pre-osteoblast cells cultured with osteogenic media was compared with negative control group (Serum Free Media). The statistical analysis showed significant difference with **p-value=0.001. n=6 (technical replicates)

6.3.1 The effect of the concentrated CM on ALP actvity of pre-osteoblastic cells

The ALP Assay was then used to analyse the effect of 3-day concentrated CM on the MC3T3 cells cultured in osteogenic media with additional of 100ug/ml protein of CM for 3 days. This experiment was performed to analyse the ALP activity of the cells after induced by the selected CM. Comparison of the ALP activity of the cells cultured in CMs plus osteogenic media, with the cells cultured with osteogenic media alone which is known to promote ALP production was undertaken. This approach was performed in order to observe any added benefit of the CMs on ALP activity of the cells. There was a further group of cells cultured in serum free media only to serve as the negative control.

Pre-osteoblasts cells (MC3T3) were seeded in 96-well plate with 10,000 cells per well in 150ul α-MEM (10% FBS). After 24 hours, the media in each plate was removed and the wells were washed three times with 100ul PBS to ensure complete removal of serum media. Then, 100ug/ml proteins of collected CM which were mixed SFM were added in the wells (5 wells per CM). The cells were incubated for another three days before the cells were lysed with 0.1% Triton x-100 and incubated with prepared p-nitrophenyl phosphate solution (Sigma-Aldsrich, UK) for 1 hour in 37°C. The result of the reaction is p-nitrophenol production, and it was quantified with automatic plate reader at the 405nm wavelength with prepared standards of p-nitrophenol.

Results showed that the CMs did not induce the pre-osteoblast cells to produce more ALP enzyme activity compared with the group with the osteogenic media only. However, all media groups produced higher ALP activity compared with serum free media.

When statistical comparisons were performed using osteogenic media as the control group, there were no significant difference found (p-value = 1.000) between all CMs. There was also no difference of the ALP activity between all CMs with 10% FBS media group. However, all CM treatments produced significantly higher ALP activity especially CMs from PDLSCs Normoxia (p-value = 0.001) when compared with cells cultured in serum free media.

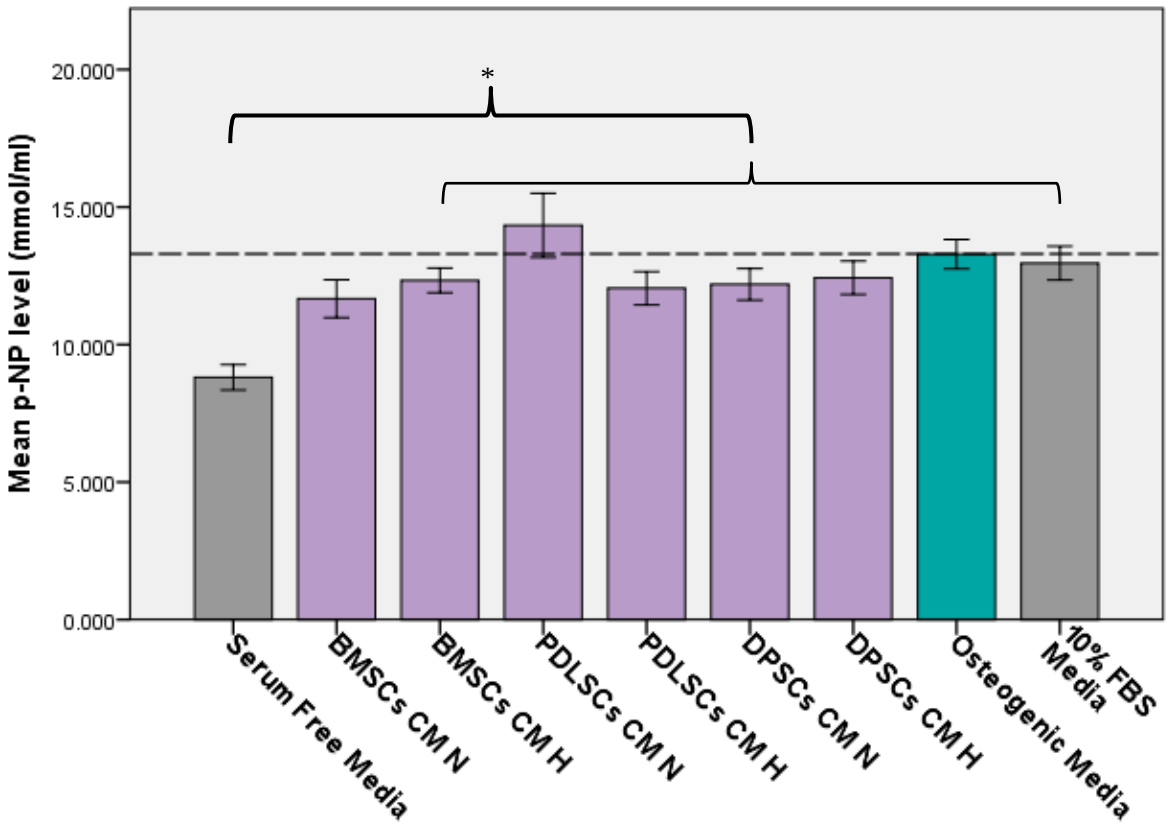


Figure 6.7 ALP activity of the cells cultured in different CM

Statistical comparison was performed between each group with negative control group (Serum Free Media group) with *p-value<0.05. No significance different was obtained when comparison was performed with the osteogenic media and 10% FBS media groups. n=5 (technical replicates)

6.4 Effect of concentrated CM on cell migration

The third part of this chapter evaluated the effect of the CM on cell migration. For this purpose, two types of migration assays were performed: the barrier assay using IBIDI inserts allowing to analyse cell migration into a cleared space and an assay using polycarbonate track-etch (PCTE) membrane (ChemoTx® Chemotaxis System, NeuroProbe) enabling to study chemotaxic cell migration.

6.4.1 Migration of the MC3T3 cultured in different cell-types' CM

The cell-free area area was created using IBIDI® inserts, where the inserts were placed in confluent monolayer cells for 24 hours before the inserts were removed and the 10% FBS culture media were replaced with culture media containg 100ug/ml protein of the CM.

The MC3T3 cells were seeded with cell density of 1 x 10^5 cells/ml in 24-well plates and the IBIDI® inserts placed in the middle of the well. The media used was primary culture media with 10% FBS and the cultures were incubated for 24 hours in normal incubator. The media was later changed to 100ug/ml proteins of collected CM plus SFM. The inserts were also removed, and the image of the gap created by the inserts between cells confluent monolayer were photographed at the middle of each well. The gap measured using Image J software as the baseline data. Then, the cultures were incubated again for another 24 hours before the gap between cells in each group was re-measured. The edges of the gap in the images were carefully outlined and the area of the outlined area were automatically analysed by the software.

At the beginning of the experiment, there was no difference of the surface area which was not covered by the cells in all groups as shown in Figure 6.8 and Table 6-1. After 24 hours, the gap was reaching closure in the FBS cultures but was still apparent in the other (SF) groups. The gap remaining in the PDL CM group was the smallest with significant statistical difference from the SFM group (p-value <0.001). Despite the difference in the mean area of gap between BM CM and DP CM groups with the SFM group, the differences were not statistically significant.

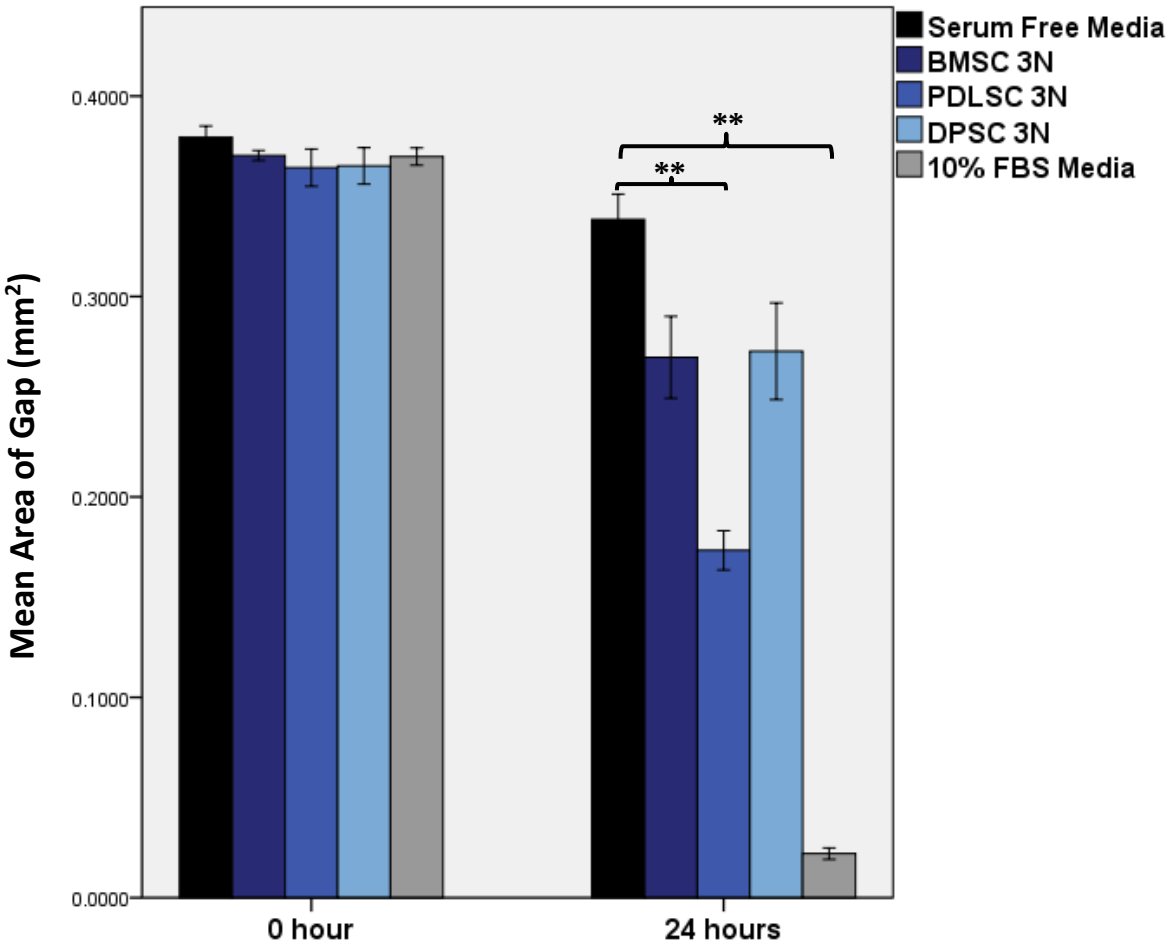
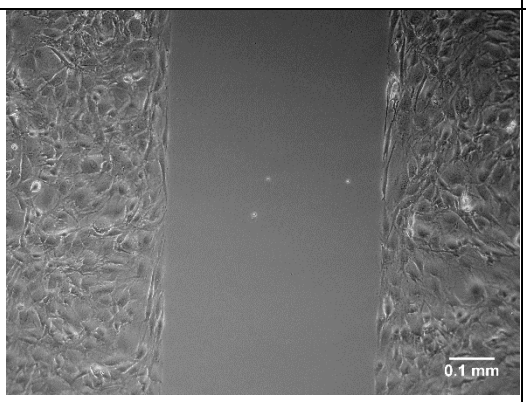
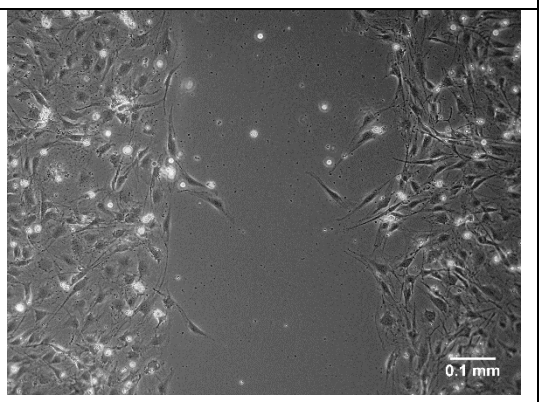
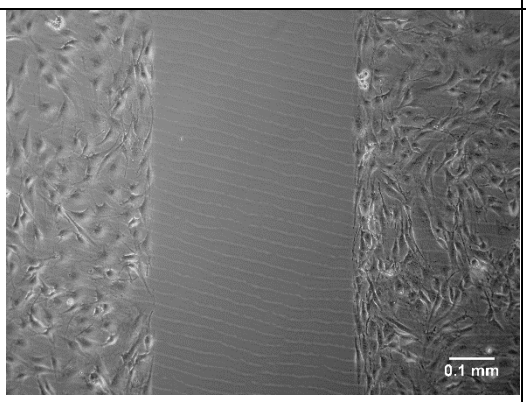
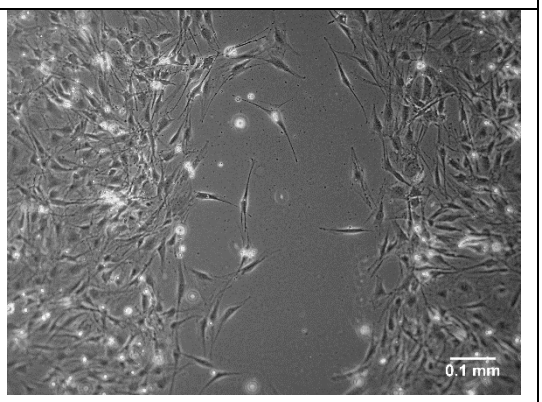
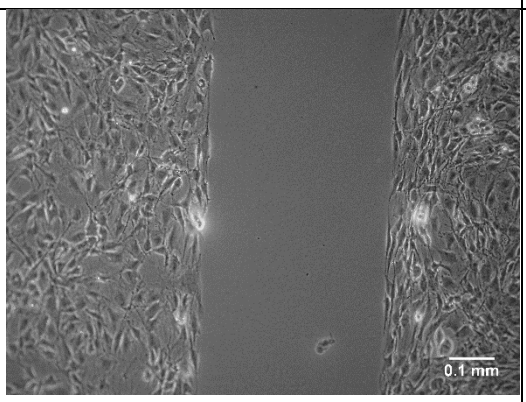
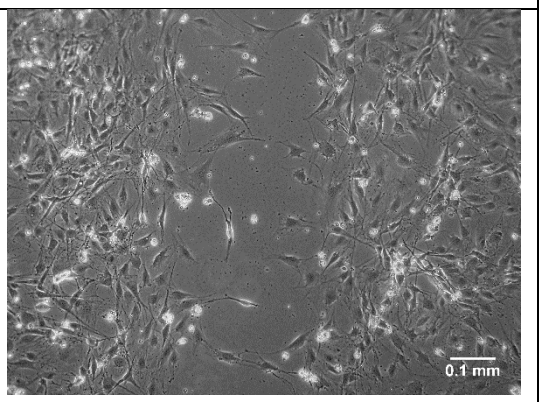
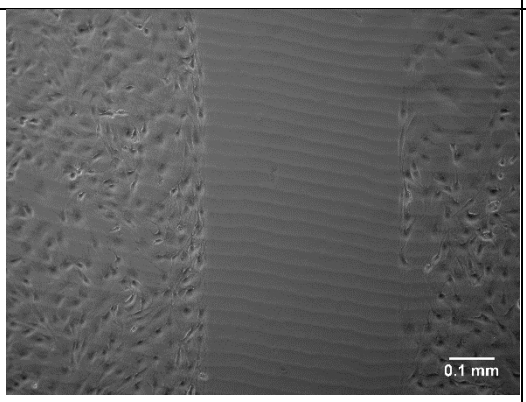
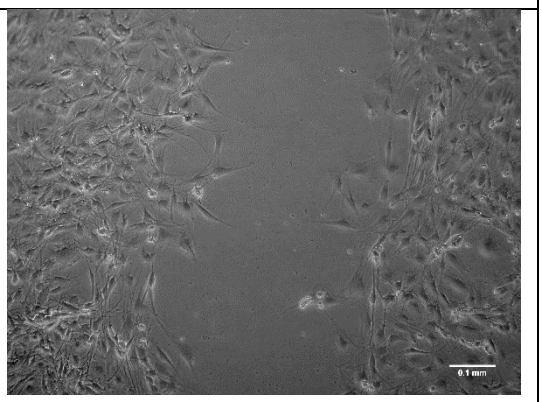


Figure 6.8 Scratch Assay of pre-osteoblast cells cultured in different CM

Statistical comparison was performed between each group with negative control group (Serum Free Media group) with **p-value=0.001. No significance different was obtained at 0 hour. n=6.

	Mean Area (mm^2)						
	SFM	BM3N	p-value	PDL 3N	p-value	DP3N	p-value
0 hour	0.380±0.014	0.370±0.006	1.000	0.364±0.023	1.000	0.365±0.022	1.000
24 hours	0.339±0.031	0.270±0.050	0.052	**0.173±0.024**	**<0.001**	0.273±0.059	0.072

Table 6-1 The area of the surfaces covered by the migrated cells after 24 hours in different CM compared to cells cultured in serum free media (SFM). n=6 (technical replicates)

	0 hour	24 hours
SFM		
BM 3N		
PDL 3N		
DP 3N		

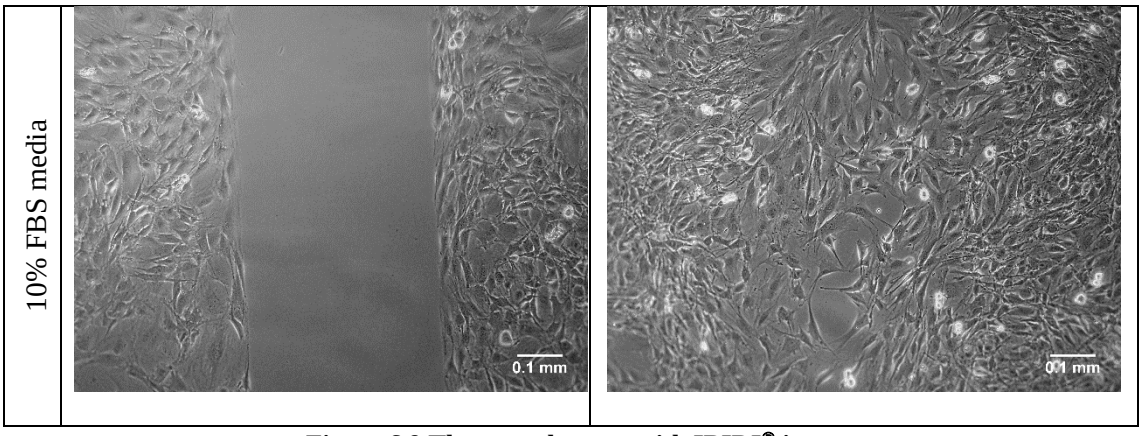
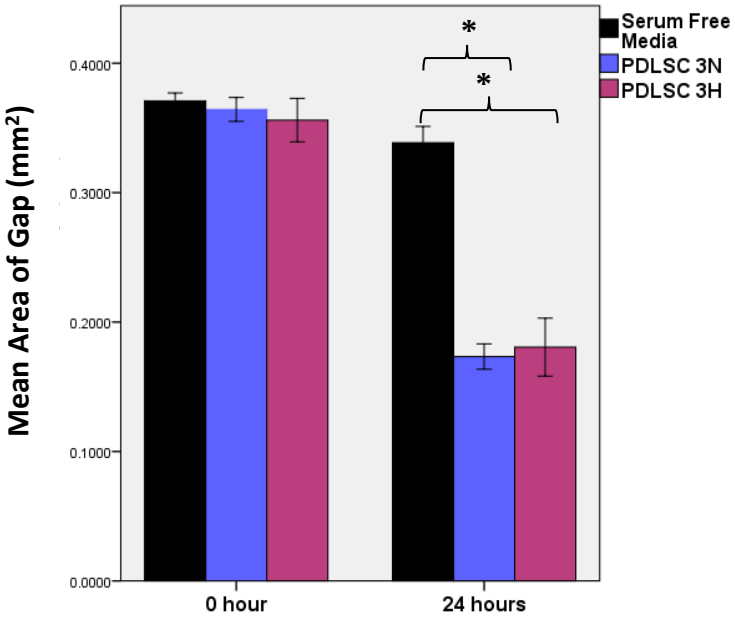
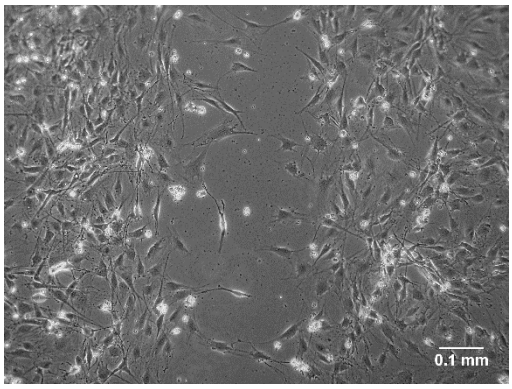
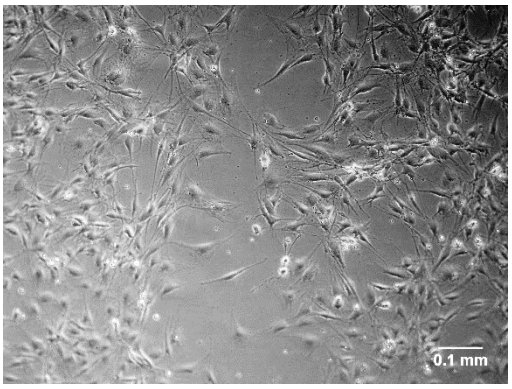


Figure 6.9 The scratch assay with IBIDI® inserts

The phase-contrast images of the pre-osteoblast cells monolayers at the beginning of the experiment and after 24 hours. Scaler bar represents 0.1mm in 10x magnification. n=6 (technical replicates).

Next the difference in the cell migration was studied after treatment with hypoxia or normoxia CM. The result showed that mean remaining areas were reduced from 0.364 ±0.023mm^2 to 0.1733 ±0.023 mm^2 when the cells were cultured in normoxia CM and from 0.4370 ±0.04mm^2 to 0.1807 ±0.055 mm^2 when cultured in hypoxia CM. Thus, there was no difference in migration of the cells when cultured in normoxia or hypoxia CM.



	PDL 3N	PDL 3H
24 hours		

(B)

Figure 6.10 Scratch Assay of pre-osteoblast cells cultured in normoxia and hypoxia CM. Area of gap between cells before and after 24 hours of migration of the pre-osteoblast cells. Statistical comparison was performed between each group with negative control group (Serum Free Media group) with **p-value=0.001. No significance different was obtained at 0 hour. N=6 (technical replicates). (B) Images of the monolayer cells after 24 hours. Scale bar represents 0.1mm in 10x magnification.

6.4.2 Transmembrane migration assay

The second experiment for evaluating the ability of the CM to promote osteoblast migration was using the ChemoTx transmembrane. The cells were placed on the membrane with 20µl of serum-free media and the membrane was placed into a well which was filled with 100µg/ml of CM with serum free media of total 150µl per well. The experiment was left for 24 hours before the migrated cells were labelled with Calcein and the fluorescence of the media containing the calcein labelled-migrated cells were measured with fluorecence spectrometer.

No significant difference was found between all CM groups as compared to negative control group as shown in Figure 6.11. However, the difference of the the positive group with the CM groups and negative control was significant. Even with increased protein concentration (ie 200 and 300 µg/ml). The data failed to show significant differences in transmembrane cell migration (Figure 6.12).

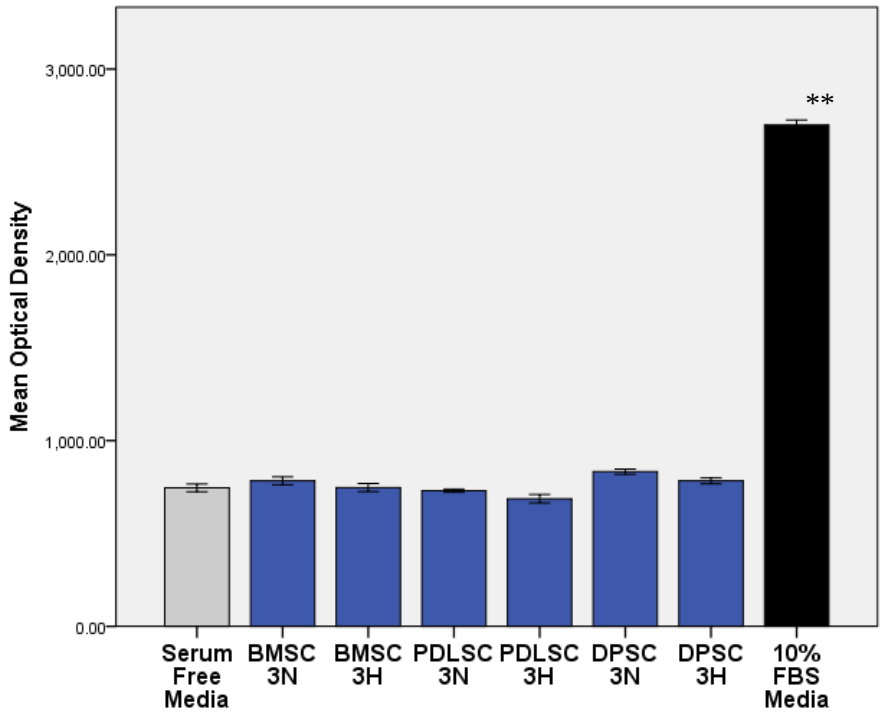


Figure 6.11 Transmembrane Migration Assay

Statistical comparison was performed between each group with negative control group (Serum Free Media group). No significance different was obtained. When comparison was performed with the 10% FBS media, all group showed significant difference with **p-value=0.001.

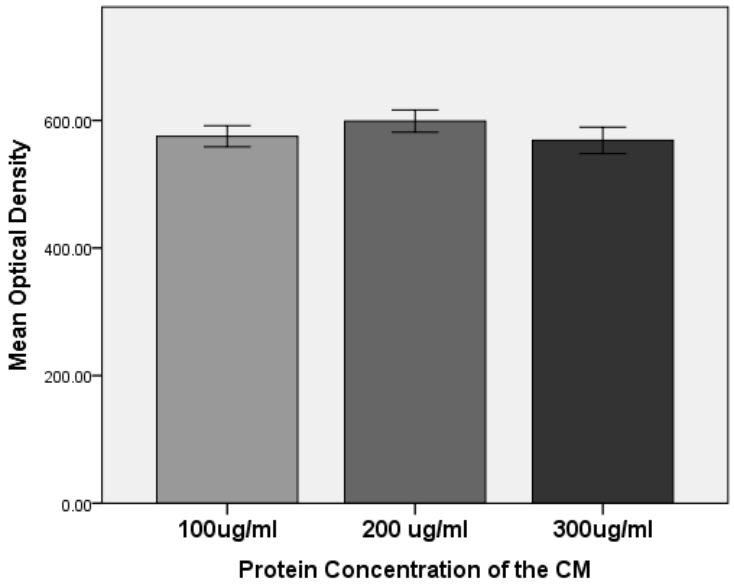


Figure 6.12 Transmembrane migration assay with different protein concentrations

Statistical comparison was performed between 200µg/ml and 300µg/ml of protein in the PDLSC CM with '100ug/ml group' served as negative control group. No significance different was obtained. N=8

6.5 Discussion

6.5.1 Effect of CM on MSC metabolic activity

One important experiment to evaluate the effectiveness of secretomes on other cells is by evaluating the target cells metabolic activity. MTT assay is one of the mostly widely used method for this purpose, even though MTT staining only indirectly reflects mitochondrial activity and often reflects the number of viable cells after culture so is mainly used in either cell toxicity or cell growth studies. Several other secretome studies used MTT assay in evaluating the effect of the CMs on other cells (Ogata, Katagiri et al. 2015, Wang, Zhou et al. 2015, Xia, Tang et al. 2016, Xu, Wang et al. 2016). However, the amount of the CM used in those studies varied; with some studies diluting their secretome (Paschalidis, Bakopoulou et al. 2014, Ogata, Katagiri et al. 2015, Xia, Tang et al. 2016, Kumar, Kumar et al. 2017) while some utilise specific protein concentration of the CM (Wang, Zhou et al. 2015, Xu, Wang et al. 2016). The majority of the other studies did not report in detail on how the CMs were used (Hung, Pochampally et al. 2007, Aranha, Zhang et al. 2010, Osugi, Katagiri et al. 2012, Inukai, Katagiri et al. 2013, Mita, Furukawa-Hibi et al. 2015, Tachida, Sakurai et al. 2015, Ratajczak, Hilkens et al. 2016) and were presumed to used undiluted CM for the experiments.

Despite the different methods used, most of the studies reported that applied MSC CMs increased target cell metabolic activitiy. One study reported that dilution of the CMs produced better cell metabolic activity compared with the undiluted CM (Paschalidis, Bakopoulou et al. 2014) although another study reported that 2-fold and 4-fold dilutions of the CM did not give any significant changes in cell viability (Ogata, Katagiri et al. 2015).

With regard to different protein concentrations of the CMs, one study did not find any positive effect on cell activity using MTT assay when different protein concentrations of the CMs from human umbical stem cells were applied. The protein concentrations used in that specific study were between 0.5µg/ml to 20µg/ml (Wang, Xu et al. 2015). Notably, increased protein concentrations of the human BMSC CM to 100µg/ml and 200µg/ml did not give any significant difference in cell viability,

however the researchers demonstrated that a 100µg/ml protein concentration could stimulate mineralization in osteogenic differentiation assay (Xu, Wang et al. 2016).

Thus, in this current a 100µg/ml protein concentration of CMs was used in proliferation assay with MTT and increased cell activity could be observed than the one in serum free media which served as negative control. Increasing protein concentration to 200 µg/ml did result in increased cellular activity, albeit statistically insignificant.

Findings presented here also showed that applying a 100µg/ml concentration of each CM could maintain cell viability for up to two days, but cell activity was found to be reduced by the third day in all CM samples which was similar to the findings reported in Ratajczak et al. They found that the CM from dental MSCs was not able to increase the proliferation of endothelial cells and this decreased after 3 days in their study (Ratajczak, Hilkens et al. 2016). This implies that for future experiment, the media with the CM should be refreshed every two days to avoid deterioration of cell activity. Although one study reported that the cell viability was higher on the fifth day compared to the third day when cells were cultured with 20µg/ml CM (Wang, Xu et al. 2015). Another study reported that the PDLSC CM could maintain cell viability of PDLSCs for up to seven days (Xia, Tang et al. 2016). However the possible reason for the positive response of the cell viability in this study was sue to the CM being used being derived from the same cell line that produced the CM (PDLSCs CM on PDLSCs cell), which was in contrast to the study presented here that used pre-osteoblast cells. Thus, the effect of the CMs on the same cells (autocrine) or other cells (paracrine) is worthy to be studied in future in *in vitro* experiments.

Another part of this study found that the effect of CM on cell metabolic activity was also determined by the days of the CM collection. Most of the secretome studies collected their CMs after the first or second day of culture and showed positive effects on cell viability when the CMs were tested on other cell types (Hung, Pochampally et al. 2007, Chen, Tredget et al. 2008, Aranha, Zhang et al. 2010, Osugi, Katagiri et al. 2012, Inukai, Katagiri et al. 2013, Mita, Furukawa-Hibi et al. 2015, Ogata, Katagiri et al. 2015, Wang, Xu et al. 2015, Ratajczak, Hilkens et al. 2016, Xu, Wang et al.

2016, Kumar, Kumar et al. 2017). Studies that collected the CMs from longer durations are limited (Paschalidis, Bakopoulou et al. 2014, Tachida, Sakurai et al. 2015, Xia, Tang et al. 2016). This study provided additional data on longer duration of CMs collected. In fact, it was found that the longer duration of CM collection would produce a more potent secretome for cell viability; specifically, for BMSC's CM from both normoxia and hypoxia culture conditions. This finding was strengthened by our earlier result that showed higher growth factor level, especially TGF-β1 in BMSC's CM from the third day collection compared to the second day. As mentioned earlier, TGF-β1 is one of the growth factors that may promote stem cell proliferation (Watabe and Miyazono 2009).

The data on PDLSCs' CM different day collections showed that third day CM is more effective in stimulating cell viability compared with second day CM but only when the PDLSCs cultured in normal oxygen concentration. Furthermore, the TMT mass spectrometry data could not identify any specific growth factors or proteins that increased in Day 2 CM (hypoxia). Furthermore, investigation of other growth factors should be elucidated to confirm this finding.

6.5.2 Effect of CM on cellular ALP activity

Alkaline phosphatase is an enzyme produced by osteoblast-like cells that is considered to be a key the marker for osteogenic/osteoblast differentiation (Golub and Boesze-Battaglia 2007). it was hypothesized that the MSC secretome in the CM would cause increased ALP production by these cells. Unfortunately, this study indicated that the CM did not give any added benefit in inducing pre-osteoblast cell differentiation compared with osteogenic media only as measured by the ALP activity. This finding is not in-line with the results from another studies in which human BMSCs were cultured in osteogenic media with CM from BMSC (Xu, Wang et al. 2016).

This is probably due to a longer duration of the differentiation experiment which was 7 days compared to 3 days in our experiment. We used 100 µg/ml of CM which was on average around 6% to 7% of total osteogenic media. The protein concentration of 100µg/ml from CM have been found

to be the optimal concentration for mineralisation compared to lower (10, 25, 50µg/ml) or higher (200µg/ml) (Xu, Wang et al. 2016).

Another important aspect of the results that worth to be discussed is the usage of MC3T3 cells in the experiments. The cells have been shown to be actively proliferating at initial culture (up to 9 days) and produce limited ALP. It was stated that the expression of ALP by MC3T3 was time dependent and usually takes up to 16 days in culture (Quarles, Yohay et al. 1992). This reflected the result as presented in section 6.3.1, in which neither CMs nor the osteogenic media abled to induce ALP production by the cells .

Thus, improvement of the experiment should include longer duration of the differentiation time and/or usage of the undifferentiated cells to test the CM potential. The CMs should also be tested in the *in vitro* mineralisation assay with identification of specific osteogenic differentiation markers such as *Runx* or *core-binding factor subunit alpha 1(cbfa-1)* which is known as the key regulator for osteoblast differentiation (Ducy 2000). Other than that, late osteogenic markers such as *Osteocalcin* which is one of the extracellular matrix proteins in bone should also be incorporated to strengthen the findings (Rutkovskiy, Stensløkken et al. 2016).

6.5.3 Effect of CM on cell migration

One of the main components in tissue regeneration is the migration of the progenitor cells from the surrounding area to the regeneration site, based on the appropriate stimuli or chemotactic factors. Thus, the experiments on the effect of the CMs on cell migration ability were divided into two main components. First, to test the effect of the CMs on the cells migratory ability and secondly to evaluate the chemotactic signal of the CMs.

For the initial study, the IBIDI® inserts were used to create gaps between cells and cells were cultured with the CM. Cell migration was stimulated in all groups compared with the negative control group

specifically the group which was cultured with PDLSC CM. The ability of cell migration in BMSC CM and DPSC CM groups were similar.

These findings may possibly be linked to the higher level of IGF-1 in PDLSC CM compared with BMSC CM and DPSC CM (Chapter 4). Many studies have associated the IGF-1 not only as a potent mitogen but as a cell migratory inducer of mesenchymal stem cells (Li, Yu et al. 2007, Huang, Qiu et al. 2012, Xinaris, Morigi et al. 2013), endothelial cells (Shigematsu, Yamauchi et al. 1999) and ovarian cells(Ukaji, Lin et al. 2015). IGF-1 induced cell migration by increasing the expression of CXC chemokine receptor 4 (CXCR4) on the cell surface (Xinaris, Morigi et al. 2013).

Another study that used dental secretome also showed similar finding in a scratch assay experiment. where the migration was reported to be higher when the CM was tested on rat PDLSCs compared with BMSCs (Kawai, Katagiri et al. 2015).

Data presented here also found that hypoxic CM did not increase cell migratory activity compared with normoxic CM. This was not consistent with another study which found hypoxic CM of BMSCs could increase BMSC migration compared with normoxic CM (Chang, Chio et al. 2013). However, the level of oxygen that was used was only 0.5% compared to 2% as used in this study. These variabilities made the comparison between studies difficult as no standardized methods were used.

The second approach used the transmembrane assay system to evaluate the chemotactic ability of the CM. None of our CMs promoted migration of the pre-osteoblast cells across the membrane. This finding sems contradictory to the barrier assay and as reported in other studies using dental stem cells (Osugi, Katagiri et al. 2012, Inukai, Katagiri et al. 2013, Paschalidis, Bakopoulou et al. 2014, Ratajczak, Hilkens et al. 2016). This may be because different cell types were used. Furthermore, those studies were using Day 1 or Day 2 CM, however Day 3 CM was used here which may result in some of the chemokines in the CMs being degraded due to the extended incubation time (Chen, Tredget et al. 2008, Osugi, Katagiri et al. 2012, Inukai, Katagiri et al. 2013, Ratajczak, Hilkens et al. 2016). The chemokines involved worth to be elucidated in future studies. One previous study used CM of longer than 3 days culture duration, but the experiment was performed with 0.5% FBS in the

CM and the difference between the CM and negative control was not statistically significant (Paschalidis, Bakopoulou et al. 2014).

Another possible reason for the negative chemotactic result in this study was probably due to the inadequate amount of CM per well in this experiment that could stimulate chemotaxis. Indeed 100µg/ml of protein CM was used which is equivalent to 5-7% of total media volume per well. The majority of other studies have demonstrated positive results in transmembrane migration assays but did not report the amount of CM used in their experiments (Osugi, Katagiri et al. 2012, Inukai, Katagiri et al. 2013) except two studies that used 12.5% to100% CM in their media (Chen, Tredget et al. 2008, Paschalidis, Bakopoulou et al. 2014).

6.5.4 Conclusion

In conclusion, the results of this chapter provide new evidence that CMs from the three different MSCs produced similar effects on pre-osteoblast cell metabolic activity, ALP activity and migration. In particular, the MSC-CM could promote pre-osteoblast cells viability in the short-term duration.

CHAPTER 7

GENERAL DISCUSSION

7 GENERAL DISCUSSION AND CONCLUSION

Mesenchymal stem cells have been found in various tissues in the body and each respective stem cell has unique characteristics although they are reported to share the same stem cell markers and differentiation ability. This PhD study isolated the stem cells of the PDLSCs and DPSCs to compare their characteristics with BMSCs.

This study noted that the PDLSCs have lower osteogenic potential compared with BMSCs and DPSCs based on the formation of calcified nodules after osteogenic differentiation assay. Even the expression of osteogenic genes such as *Runx/cbfa-1* and *ALP* by the PDLSCs was also lower compared with BMSCs and DPSCs. Another part of the study which was performed in order to fulfil the criteria by ISCT for MSCs was the adipogenic differentiation assay. Although, the three MSCs showed evidence of oil doplets in the adipogenic differentiation assay, the result would be strengthen with adipogenic gene expression studies such as analyses of *perioxisome proliferator-activated receptor gamma (PPAR-γ), fatty acid binding protein 4 (FACB-4)* and *CCAAT/ enhancer-binding protein alpha (C/EBP-α*) (Munir, Ward et al. 2017). For future studies, these genes should be incorporated together with adipogenic differentiation assay.

The data also indicated that, dental-derived stem cells samples produced lower gene expression for *VEGF* and *TGF-β1* compared with BMSCs. Interestingly, IGF-1 was also detected in the PDLSC secretome and confirmed by gene expression analysis. The undetected IGF-1 protein in the CM of BMSCs and DPSCs was probably due to the presence of IGF-BP1 which was expressed at higher levels by these cell types.

The data presented in this thesis indicated that lower oxygen concentration could affect the growth factor production by the MSCs. Incubation with lower oxygen concentration caused the three MSCs to to produce the secretome with increase levels of the VEGF and TGF-β1 although the gene expression analysis showed similar levels between hypoxia and normoxia incubations. Interestingly,

IGF-1 was only produced by PDLSCs in this study via protein assay. Although data also showed the evidence of *IGF-1* expression in all three cell lines, PDLSCs expressed the highest level of the gene especially under hypoxic conditions.

There were no obvious changes in other protein levels which were detected in quantitative mass spectrometry (via TMT analysis); only a small number of proteins were identified in this analysis. From the mass spectrometry data, the list of proteins secreted by MSCs were numerous and the quantity of proteins were different between each cell-line with BMSCs producing the most proteins and PDLSCs the least. The similarity of the proteins secreted by the BMSCs and DPSCs were higher compared with the one with PDLSCs. This could provide as a protein inventory for the MSCs secretome and is worthy deliberation in future studies.

This study also found that different collection days for the CMs could produce different levels of the growth factors. Longer cell culture (three days) produced increased level of VEGF, TGF-β1 and IGF-1 compared with two days. Furthermore, filtration with 0.2μm filter of the CM could reduce the level of VEGF, TGF-β1 and IGF-1.

The proliferation of the pre-osteoblast cells was increased in the presence of the MSC secretomes compared with negative controls, but there was no positive effect on ALP activity in the pre-osteoblast cells. This is probably due to the duration of the experiment which was only for three days for the significant difference to be observed. There were also no positive effects of the secretome on cell migration except for the secretome from PDLSCs. Also, there was no difference in migratory effect between normoxic and hypoxic samples.

One of the challenges in doing this research study was to culture and grow the dental-derived MSCs. This is because as the cells were harvested from rats, the number of cells obtained at passage 0 was minimal and it takes significant time for cell expansion. Furthermore, these primary cells were also sensitive; easily infected or died due to changes in the culture condition.

7.1 Future recommendations

The proteomic analysis of the secretomes in CMs in this study were limited to ELISA of three main growth factors along with mass spectrometry analysis. Quantitative mass spectrometry was considered as preliminary or exploratory because limited proteins were successfully tagged for quantification. Thus, future study should include more comprehensive proteomic analysis such as ELISA microarray and analysing more samples for mass spectrometry.

As this PhD project focused on the effect of the the secretome in the CMs to the pre-osteoblast cell proliferation, ALP activity and migration, further research is needed to explore the usage of the collected CM on other important functional assays such as in angiogenic and mineralisation assays and compare outcomes between BMSCs, PDLSCs and DPSCs secretome samples. The effect of the CMs on the immunomodulatory reactions of the lymphocyte cells should also be evaluated to test whether the CMs could elicit any immunostimulatory or immunosupression reactions.

The usage of the CM should also be tested on the MSCs themselves suc to mimic the target cells present in *in vivo* conditions. Furthermore, different CM concentrations should be used over a wider range in order to detect significant difference in various functional assays.

Another area worthwhile to be explored is the usage of the dental-derived secretomes with scaffolds specifically using the nanotechnology example carbon nanotubes in order to improve the findings in tissue engineering research. In fact multi-walled carbon nanotubes have been used together with the MSCs from human and dogs in a few *in vitro* studies and there were increased osteogenic and neuronal differentiation of the MSCs compared with the MSCs without the carbon nanotubes (Nayak, Jian et al. 2010, Das, Madhusoodan et al. 2017). Thus, the influence of the carbon nanotubes to the secretome would be an interesting area to be explored further in future studies.

7.2 Conclusion

In conclusion, dental-derived secretomes of PDLSCs and DPSCs have the potential to be potential biological mediators for tissue regeneration as secretome from BMSCs because the secretomes contain numerous proteins that are indiscriminate for any particular cell type and their content could be altered with various culture conditions such as hypoxia incubation, different collection day of the secretomes and secretome filtration. Although the secretome contain complex and diverse mixture of proteins, the secretomes from PDLSCs and DPSCs produced similar effect on cell activities as the secretome of BMSCs. The study of the dental-derived secretomes is still considered early and there are many more gaps to be explored which could provide interesting future research studies.

8 REFERENCES

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Agata, H., H. Kagami, N. Watanabe and M. Ueda (2008). "Effect of ischemic culture conditions on the survival and differentiation of porcine dental pulp-derived cells." Differentiation **76**(9): 981-993.

Ahmed, N. E.-M. B., M. Murakami, Y. Hirose and M. Nakashima (2016). "Therapeutic Potential of Dental Pulp Stem Cell Secretome for Alzheimer's Disease Treatment: An In Vitro Study." Stem Cells International **2016**.

Ahmed, N. E.-M. B., M. Murakami, S. Kaneko and M. Nakashima (2016). "The effects of hypoxia on the stemness properties of human dental pulp stem cells (DPSCs)." Scientific reports **6**: 35476-35476.

Al-Sharabi, N., M. Mustafa, M. Ueda, Y. Xue, K. Mustafa and I. Fristad (2016). "Conditioned medium from human bone marrow stromal cells attenuates initial inflammatory reactions in dental pulp tissue." Dental Traumatology: <xocs:firstpage xmlns:xocs=""/>.

Alkharobi, H. E., H. Al-Khafaji, J. Beattie, D. A. Devine and R. El-Gendy (2018). "Insulin-Like Growth Factor Axis Expression in Dental Pulp Cells Derived From Carious Teeth." Frontiers in bioengineering and biotechnology **6**: 36-36.

Alvarez-Llamas, G., E. Szalowska, M. P. de Vries, D. Weening, K. Landman, A. Hoek, B. H. Wolffenbuttel, H. Roelofsen and R. J. Vonk (2007). "Characterization of the human visceral adipose tissue secretome." Mol Cell Proteomics **6**(4): 589-600.

Amemiya, H., K. Matsuzaka, E. Kokubu, S. Ohta and T. Inoue (2008). "Cellular responses of rat periodontal ligament cells under hypoxia and re-oxygenation conditions in vitro." Journal of Periodontal Research **43**(3): 322-327.

Amemiya, K., Y. Kaneko, T. Muramatsu, M. Shimono and T. Inoue (2003). "Pulp cell responses during hypoxia and reoxygenation in vitro." European Journal of Oral Sciences **111**(4): 332-338.

Amemiya, K., Y. Kaneko, T. Muramatsu, M. Shimono and T. Inoue (2003). "Pulp cell responses during hypoxia and reoxygenation in vitro." Eur J Oral Sci **111**(4): 332-338.

Ando, Y., K. Matsubara, J. Ishikawa, M. Fujio, R. Shohara, H. Hibi, M. Ueda and A. Yamamoto (2014). "Stem cell-conditioned medium accelerates distraction osteogenesis through multiple regenerative mechanisms." Bone **61**(0): 82-90.

Aranha, A. M., Z. Zhang, K. G. Neiva, C. A. Costa, J. Hebling and J. E. Nor (2010). "Hypoxia enhances the angiogenic potential of human dental pulp cells." J Endod **36**(10): 1633-1637.

Aranha, A. M. F., Z. Zhang, K. G. Neiva, C. A. S. Costa, J. Hebling and J. E. Nör (2010). "Hypoxia Enhances the Angiogenic Potential of Human Dental Pulp Cells." Journal of Endodontics **36**(10): 1633-1637.

Asahara, T., H. Masuda, T. Takahashi, C. Kalka, C. Pastore, M. Silver, M. Kearne, M. Magner and J. M. Isner (1999). "Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization." Circulation research **85**(3): 221.

Aukhil, I., D. M. Simpson, C. Suggs and E. Pettersson (1986). "In vivo differentiation of progenitor cells of the periodontal ligament. An experimental study using physical barriers." Journal of Clinical Periodontology **13**(9): 862-868.

Bakopoulou, A., A. Kritis, D. Andreadis, E. Papachristou, G. Leyhausen, P. Koidis, W. Geurtsen and A. Tsiftsoglou (2015). "Angiogenic Potential and Secretome of Human Apical Papilla Mesenchymal Stem Cells in Various Stress Microenvironments." Stem Cells Dev **24**(21): 2496-2512.

Barber, R. D., D. W. Harmer, R. A. Coleman and B. J. Clark (2005). "GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues." Physiol Genomics **21**(3): 389-395.

Bartold, P. M. and S. Gronthos (2017). "Standardization of Criteria Defining Periodontal Ligament Stem Cells." Journal of Dental Research **96**(5): 487-490.

Bartold, P. M., S. Gronthos, S. Ivanovski, A. Fisher and D. W. Hutmacher (2016). "Tissue engineered periodontal products." J Periodontal Res **51**(1): 1-15.

Bendall, S. C., M. H. Stewart, P. Menendez, D. George, K. Vijayaragavan, T. Werbowetski-Ogilvie, V. Ramos-Mejia, A. Rouleau, J. Yang, M. Bossé, G. Lajoie and M. Bhatia (2007). "IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro." Nature **448**: 1015.

Bianco, P., M. Riminucci, S. Gronthos and P. G. Robey (2001). "Bone marrow stromal stem cells: Nature, biology, and potential applications." Stem Cells **19**(3): 180-192.

Bongso, A. and M. Richards (2004). "History and perspective of stem cell research." Best Pract. Res. Clin. Obstet. Gynaecol. **18**(6): 827-842.

Celebi, B., A. E. Elcin and Y. M. Elcin (2010). "Proteome analysis of rat bone marrow mesenchymal stem cell differentiation." J Proteome Res **9**(10): 5217-5227.

Chang, C.-P., C.-C. Chio, C.-U. Cheong, C.-M. Chao, B.-C. Cheng and M.-T. Lin (2013). "Hypoxic preconditioning enhances the therapeutic potential of the secretome from cultured human mesenchymal stem cells in experimental traumatic brain injury." Clinical Science **124**(3): 165-176.

Chen, F.-M., L.-N. Gao, B.-M. Tian, X.-Y. Zhang, Y.-J. Zhang, G.-Y. Dong, H. Lu, Q. Chu, J. Xu, Y. Yu, R.-X. Wu, Y. Yin, S. Shi and Y. Jin (2016). "Treatment of periodontal intrabony defects using autologous periodontal ligament stem cells: a randomized clinical trial." Stem Cell Research & Therapy **7**(1): 33.

Chen, L., E. E. Tredget, P. Wu and Y. Wu (2008). "Paracrine Factors of Mesenchymal Stem Cells Recruit Macrophages and Endothelial Lineage Cells and Enhance Wound Healing." PLoS One **3**(4).

Chen, S., W. Fang, F. Ye, Y. Liu, J. Qian, S. Shan, J. Zhang, R. Zhao, L. Liao, S. Lin and J. P. Sun (2004). "Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction." Am. J. Cardiol. **94**(1): 92-95.

Choi, H., H. Jin, J.-Y. Kim, K.-T. Lim, H.-W. Choung, J.-Y. Park, J. H. Chung and P.-H. Choung (2014). "Hypoxia promotes CEMP1 expression and induces cementoblastic differentiation of human dental stem cells in an HIF-1-dependent manner." Tissue engineering. Part A **20**(1-2): 410.

Choi, M.-H., W.-C. Noh, J.-W. Park, J.-M. Lee and J.-Y. Suh (2011). "Gene expression pattern during osteogenic differentiation of human periodontal ligament cells in vitro." Journal of Periodontal & Implant Science **41**(4): 167-175.

Cornish, J., A. Grey, K. E. Callon, D. Naot, B. L. Hill, C. Q. X. Lin, L. M. Balchin and I. R. Reid (2004). "Shared pathways of osteoblast mitogenesis induced by amylin, adrenomedullin, and IGF-1." Biochemical and biophysical research communications **318**(1): 240-246.

Curtis, T. M., J. M. Hannett, R. M. Harman, N. A. Puoplo and G. R. Van de Walle (2018). "The secretome of adipose-derived mesenchymal stem cells protects SH-SY5Y cells from arsenic-induced toxicity, independent of a neuron-like differentiation mechanism." Neurotoxicology **67**: 54-64.

D'Ippolito, G., S. Diabira, G. A. Howard, B. A. Roos and P. C. Schiller (2006). "Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells." Bone **39**(3): 513-522.

D'Ippolito, G., S. Diabira, G. A. Howard, B. A. Roos and P. C. Schiller (2006). "Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells." Bone **39**(3): 513-522.

Das, K., A. P. Madhusoodan, B. Mili, A. Kumar, A. C. Saxena, K. Kumar, M. Sarkar, P. Singh, S. Srivastava and S. Bag (2017). "Functionalized carbon nanotubes as suitable scaffold materials for proliferation and differentiation of canine mesenchymal stem cells." Int J Nanomedicine **12**: 3235-3252.

Davies, O. G., P. R. Cooper, R. M. Shelton, A. J. Smith and B. A. Scheven (2015). "A comparison of the in vitro mineralisation and dentinogenic potential of mesenchymal stem cells derived from adipose tissue, bone marrow and dental pulp." J Bone Miner Metab **33**(4): 371-382.

Davies, O. G., P. R. Cooper, R. M. Shelton, A. J. Smith and B. A. Scheven (2015). "Isolation of adipose and bone marrow mesenchymal stem cells using CD29 and CD90 modifies their capacity for osteogenic and adipogenic differentiation." Journal of Tissue Engineering **6**: 2041731415592356.

Davies OG, C. P., Shelton RM, Smith AJ, Scheven BA. (2014). "A comparison of the in vitro mineralisation and dentinogenic potential of mesenchymal stem cells derived from adipose tissue, bone marrow and dental pulp." Journal of Bone and Mineral Metabolism **Epub**.

Dominici, M., K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. C. Marini, D. S. Krause, R. J. Deans, A. Keating, D. J. Prockop and E. M. Horwitz (2006). "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement." Cytotherapy **8**(4): 315-317.

Dou, L., Y. Wu, Q. Yan, J. Wang, Y. Zhang and P. Ji (2017). "Secretome profiles of immortalized dental follicle cells using iTRAQ-based proteomic analysis." Scientific Reports **7**(1): 7300.

Ducy, P. (2000). "Cbfa1: a molecular switch in osteoblast biology." Dev Dyn **219**(4): 461-471.

Egusa, H., W. Sonoyama, M. Nishimura, I. Atsuta and K. Akiyama (2012). "Stem cells in dentistry – Part I: Stem cell sources." Journal of Prosthodontic Research **56**(3): 151-165.

Eleuterio, E., O. Trubiani, M. Sulpizio, F. Di Giuseppe, L. Pierdomenico, M. Marchisio, R. Giancola, G. Giammaria, S. Miscia, S. Caputi, C. Di Ilio and S. Angelucci (2013). "Proteome of Human Stem Cells from Periodontal Ligament and Dental Pulp." PLoS ONE **8**(8): e71101.

Estrada, R., N. Li, H. Sarojini, J. An, M. J. Lee and E. Wang (2009). "Secretome from mesenchymal stem cells induces angiogenesis via Cyr61." J Cell Physiol **219**(3): 563-571.

Feng, F., K. Akiyama, Y. Liu, T. Yamaza, T. M. Wang, J. H. Chen, B. B. Wang, G. T. Huang, S. Wang and S. Shi (2010). "Utility of PDL progenitors for in vivo tissue regeneration: a report of 3 cases." Oral Dis **16**(1): 20-28.

Ferrara, N. (2001). "Role of vascular endothelial growth factor in regulation of physiological angiogenesis." American Journal of Physiology-Cell Physiology **280**(6): C1358-C1366.

Ferrarotti, F., F. Romano, M. N. Gamba, A. Quirico, M. Giraudi, M. Audagna and M. Aimetti (2018). "Human intrabony defect regeneration with micrografts containing dental pulp stem cells: A randomized controlled clinical trial." J Clin Periodontol **45**(7): 841-850.

Friedenstein, A. J., Chailakhjan, R.K. & Lalykina, K.S. (1970). "The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells." Cell Tissue Kinet **3**: 393-403.

Fujio, M., Z. Xing, N. Sharabi, Y. Xue, A. Yamamoto, H. Hibi, M. Ueda, I. Fristad and K. Mustafa (2015). "Conditioned media from hypoxic-cultured human dental pulp cells promotes bone healing during distraction osteogenesis." J Tissue Eng Regen Med.

Gandia, C., A. Arminan, J. Garcia-Verdugo, E. Lledo, A. Ruiz, M. D. Minana, J. Sanchez-Torrijos, R. Paya, V. Mirabet, F. Carbonell-Uberos, M. Llop, J. Montero and P. Sepulveda (2008). "Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction." Stem Cells **26**(3): 638-645.

Gao, Q., A. D. Walmsley, P. R. Cooper and B. A. Scheven (2016). "Ultrasound Stimulation of Different Dental Stem Cell Populations: Role of Mitogen-activated Protein Kinase Signaling." Journal of Endodontics **42**(3): 425-431.

Gay, I., A. Cavender, D. Peto, Z. Sun, A. Speer, H. Cao and B. A. Amendt (2014). "Differentiation of human dental stem cells reveals a role for microRNA-218." Journal of Periodontal Research **49**(1): 110-120.

Gay, I., S. Chen and M. MacDougall (2007). "Isolation and characterization of multipotent human periodontal ligament stem cells." Orthodontics & Craniofacial Research **10**(3): 149-160.

Gharibi, B., S. Farzadi, M. Ghuman and F. J. Hughes (2014). "Inhibition of Akt/mTOR attenuates age-related changes in mesenchymal stem cells." Stem Cells **32**(8): 2256-2266.

Gharibi, B., M. Ghuman and F. J. Hughes (2016). "DDIT4 regulates mesenchymal stem cell fate by mediating between HIF1α and mTOR signalling." Scientific Reports **6**(1): 36889.

Giacoppo, S., S. R. Thangavelu, F. Diomede, P. Bramanti, P. Conti, O. Trubiani and E. Mazzon (2017). "Anti-inflammatory effects of hypoxia-preconditioned human periodontal ligament cell secretome in an experimental model of multiple sclerosis: a key role of IL-37." FASEB journal : official publication of the Federation of American Societies for Experimental Biology **31**(12): 5592-5608.

Golub, E. E. and K. Boesze-Battaglia (2007). "The role of alkaline phosphatase in mineralization." Current Opinion in Orthopaedics **18**(5): 444-448.

Gong, Q.-m., J.-j. Quan, H.-w. Jiang and J.-q. Ling (2010). "Regulation of the Stromal Cell–derived Factor-1α–CXCR4 Axis in Human Dental Pulp Cells." Journal of Endodontics **36**(9): 1499-1503.

Gottlow, J., S. Nyman and T. Karring (1992). "Maintenance of new attachment gained through guided tissue regeneration." Journal of Clinical Periodontology **19**(5): 315-317.

Gottlow, J., S. Nyman, T. Karring and J. Lindhe (1984). "New attachment formation as the result of controlled tissue regeneration." J Clin Periodontol **11**(8): 494-503.

Gottlow, J., S. Nyman, J. Lindhe, T. Karring and J. Wennström (1986). "New attachment formation in the human periodontium by guided tissue regeneration. Case reports." Journal of Clinical Periodontology **13**(6): 604-616.

Grayson, W. L. E. m. t. e. f. e. (2007). "Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells." Biochemical and Biophysical Research Communications **358**(3).

Graziano, A., R. d'Aquino, G. Laino, A. Proto, M. T. Giuliano, G. Pirozzi, A. De Rosa, D. Di Napoli and G. Papaccio (2008). "Human CD34+ stem cells produce bone nodules in vivo." Cell Prolif **41**(1): 1-11.

Gronthos, S., J. Brahim, W. Li, L. Fisher, N. Cherman, A. Boyde, P. Denbesten, P. G. Robey and S. Shi (2002). "Stem cell properties of human dental pulp stem cells." J. Dent. Res. **81**(8): 531-535.

Gronthos, S., M. Mankani, J. Brahim, P. G. Robey and S. Shi (2000). "Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo." Proc. Natl. Acad. Sci. U. S. A. **97**(25): 13625-13630.

Gygi, S. P., B. Rist, S. A. Gerber, F. Turecek, M. H. Gelb and R. Aebersold (1999). "Quantitative analysis of complex protein mixtures using isotope-coded affinity tags." Nature Biotechnology **17**: 994.

György, B., T. G. Szabó, M. Pásztói, Z. Pál, P. Misják, B. Aradi, V. László, É. Pállinger, E. Pap, A. Falus, E. I. Buzás, Á. Kittel and G. Nagy (2011). "Membrane vesicles, current state-of-the-art: Emerging role of extracellular vesicles." Cellular and Molecular Life Sciences **68**(16): 2667-2688.

Hadjidakis, D. J. and Androulakis, II (2006). "Bone remodeling." Ann N Y Acad Sci **1092**: 385-396.

Haynesworth, S. E., M. A. Baber and A. I. Caplan (1996). "Cytokine expression by human marrow-derived mesenchymal progenitor cells in vitro: effects of dexamethasone and IL-1 alpha." J Cell Physiol **166**(3): 585-592.

He, H., J. Yu, Y. Liu, S. Lu, H. Liu, J. Shi and Y. Jin (2008). "Effects of FGF2 and TGFβ 1 on the differentiation of human dental pulp stem cells in vitro." Cell Biology International **32**(7): 827-834.

Holzenberger, M., J. Dupont, B. Ducos, P. Leneuve, A. Géloën, P. C. Even, P. Cervera and Y. Le Bouc (2002). "IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice." Nature **421**: 182.

Huang, S., L. Xu, Y. Sun, T. Wu, K. Wang and G. Li (2015). "An improved protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow." Journal of Orthopaedic Translation **3**(1): 26-33.

Huang, W., S. Yang, J. Shao and Y.-P. Li (2007). "Signaling and transcriptional regulation in osteoblast commitment and differentiation." Frontiers in bioscience : a journal and virtual library **12**: 3068-3092.

Huang, Y.-l., R.-f. Qiu, W.-y. Mai, J. Kuang, X.-y. Cai, Y.-g. Dong, Y.-z. Hu, Y.-b. Song, A.-p. Cai and Z.-g. Jiang (2012). "Effects of insulin-like growth factor-1 on the properties of mesenchymal stem cells in vitro." Journal of Zhejiang University SCIENCE B **13**(1): 20-28.

Hughes Francis J. , T. W., Belibasakis Georgios and Martuscelli Gianluca (2006). "Effects of growth factors and cytokines on osteoblast differentiation." Periodontology 2000 **41**(1): 48-72.

Hung, S. C., R. R. Pochampally, S. C. Chen, S. C. Hsu and D. J. Prockop (2007). "Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K-Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis." Stem Cells **25**(9): 2363-2370.

Iida, K., T. Takeda-Kawaguchi, Y. Tezuka, T. Kunisada, T. Shibata and K.-i. Tezuka (2010). "Hypoxia enhances colony formation and proliferation but inhibits differentiation of human dental pulp cells." Archives of Oral Biology **55**(9): 648-654.

Inukai, T., W. Katagiri, R. Yoshimi, M. Osugi, T. Kawai, H. Hibi and M. Ueda (2013). "Novel application of stem cell-derived factors for periodontal regeneration." Biochemical and Biophysical Research Communications **430**(2): 763-768.

Ivanovic, Z. (2009). Hypoxia or in situ normoxia: The stem cell paradigm. Hoboken. **219:** 271-275.

Iwata, T., M. Yamato, K. Washio, T. Okano, I. Ishikawa, T. Ando, Z. Zhang, J. Feijen and S. Mukobata (2010). "Validation of human periodontal ligament-derived cells as a reliable source for cytotherapeutic use." Journal of Clinical Periodontology **37**(12): 1088-1099.

Janssens, S., C. Dubois, J. Bogaert, K. Theunissen, C. Deroose, W. Desmet, M. Kolantzi, L. Herbots, P. Sinnaeve, J. Dens, J. Maertens, F. Rademakers, S. Dymarkowski, O. Gheysens, J. Van Cleemput, G. Bormans, J. Nuyts, A. Belmans, L. Mortelmans, M. Boogaerts and F. Van de Werf (2006). "Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial." Lancet **367**(9505): 113-121.

Jeffrey, L. S., A. G. Carl, S. Harpreet, H. A. Tucker, P. Alexandra, J. L. Patrick, H. Shu-Ching, S. Jason and J. P. Darwin (2004). "Internalized Antigens Must Be Removed to Prepare Hypoimmunogenic Mesenchymal Stem Cells for Cell and Gene Therapy*." Molecular Therapy **9**(5): 747.

Jones, B. J. and S. J. McTaggart (2008). "Immunosuppression by mesenchymal stromal cells: From culture to clinic." Experimental Hematology **36**(6): 733-741.

Joseph, B. K., N. W. Savage, T. J. Daley and W. G. Young (1996). "In situ hybridization evidence for a paracrine/autocrine role for insulin-like growth factor-I in tooth development." Growth Factors **13**(1-2): 11-17.

Karsenty, G. (2000). "Role of Cbfa1 in osteoblast differentiation and function." Seminars in Cell & Developmental Biology **11**(5): 343-346.

Katagiri, W., M. Osugi, T. Kawai and H. Hibi (2016). "First-in-human study and clinical case reports of the alveolar bone regeneration with the secretome from human mesenchymal stem cells." Head & face medicine **12**(1): 5.

Kawai, T., W. Katagiri, M. Osugi, Y. Sugimura, H. Hibi and M. Ueda (2015). "Secretomes from bone marrow–derived mesenchymal stromal cells enhance periodontal tissue regeneration." Cytotherapy **17**(4): 369-381.

Kawasaki, T., Y. Sumita, K. Egashira, S. Ohba, H. Kagami, S. D. Tran and I. Asahina (2015). "Transient Exposure to Hypoxic and Anoxic Oxygen Concentrations Promotes Either Osteogenic or Ligamentogenic Characteristics of PDL Cells." Biores Open Access **4**(1): 175-187.

Kerkis, I., C. E. Ambrosio, A. Kerkis, D. Martins, E. Zucconi, S. A. S. Fonseca, R. M. Cabral, C. Maranduba, T. P. Gaiad, A. Morini, N. Vieira, M. P. Brolio, O. Sant'Anna, M. A. Miglino and M. Zatz (2008). "Early transplantation of human immature dental pulp stem cells from baby teeth to golden retriever muscular dystrophy (GRMD) dogs: Local or systemic?" J. Transl. Med. **6**.

Khorsand, A., M. B. Eslaminejad, M. Arabsolghar, M. Paknejad, B. Ghaedi, A. R. Rokn, N. Moslemi, H. Nazarian and S. Jahangir (2013). "Autologous dental pulp stem cells in regeneration of defect created in canine periodontal tissue." J Oral Implantol **39**(4): 433-443.

Kim, H.-S., R. G. Rosenfeld and Y. Oh (1997). "Biological roles of insulin-like growth factor binding proteins (IGFBPs)." Experimental &Amp; Molecular Medicine **29**: 85.

Kim, H., S. M. Choi and H. Kim (2013). "Mesenchymal Stem Cell-Derived Secretome and Microvesicles as a Cell-Free Therapeutics for Neurodegenerative Disorders." Tissue Eng. Regen. Med. **10**(3): 93-101.

Klevezal, G. A. and D. V. Shchepotkin (2012). "Incisor growth rate in rodents and the record of the entire annual cycle in the incisors of Marmota baibacina centralis." Biology Bulletin **39**(8): 684-691.

Kofoed, H., E. Sjøntoft, S. O. Siemssen and H. P. Olesen (1985). "Bone marrow circulation after osteotomy: Blood flow, pO 2 , pCO 2 , and pressure studied in dogs." Acta Orthopaedica **56**(5): 400-403.

Koide, M., S. Kinugawa, N. Takahashi and N. Udagawa (2010). "Osteoclastic bone resorption induced by innate immune responses." Periodontology 2000 **54**(1): 235-246.

Kumar, A., V. Kumar, V. Rattan, V. Jha and S. Bhattacharyya (2017). "Secretome Cues Modulate the Neurogenic Potential of Bone Marrow and Dental Stem Cells." Mol Neurobiol **54**(6): 4672-4682.

Kumar, A., V. Kumar, V. Rattan, V. Jha and S. Bhattacharyya (2018). "Secretome proteins regulate comparative osteogenic and adipogenic potential in bone marrow and dental stem cells." Biochimie **155**: 129-139.

Kupcova Skalnikova, H. (2013). "Proteomic techniques for characterisation of mesenchymal stem cell secretome." Biochimie **95**(12): 2196-2211.

Lavoie, J. R. and M. Rosu-Myles (2013). "Uncovering the secretes of mesenchymal stem cells." Biochimie **95**(12): 2212-2221.

Lee, C. H., E. K. Moioli and J. J. Mao (2006). "Fibroblastic differentiation of human mesenchymal stem cells using connective tissue growth factor." Conference proceedings : ... Annual International Conference of the IEEE Engineering in Medicine and Biology Society. IEEE Engineering in Medicine and Biology Society. Annual Conference **1**: 775-778.

Lee, C. H., B. Shah, E. K. Moioli and J. J. Mao (2010). "CTGF directs fibroblast differentiation from human mesenchymal stem/stromal cells and defines connective tissue healing in a rodent injury model." Journal of Clinical Investigation **120**(9): 3340-3349.

Lee, M. J., J. Kim, M. Y. Kim, Y. S. Bae, S. H. Ryu, T. G. Lee and J. H. Kim (2010). "Proteomic analysis of tumor necrosis factor-alpha-induced secretome of human adipose tissue-derived mesenchymal stem cells." J Proteome Res **9**(4): 1754-1762.

Lee, S., S. Jin, J. Song, K. Seo and K. Cho (2012). "Paracrine Effects of Adipose-Derived Stem Cells on Keratinocytes and Dermal Fibroblasts." Ann. Dermatol. **24**(2): 136-143.

Lennon, D. P., J. M. Edmison and A. I. Caplan (2001). "Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis." Journal of cellular physiology **187**(3): 345.

Lesley, J., V. C. Hascall, M. Tammi and R. Hyman (2000). "Hyaluronan binding by cell surface CD44." J Biol Chem **275**(35): 26967-26975.

Li, Y., X. Yu, S. Lin, X. Li, S. Zhang and Y. H. Song (2007). "Insulin-like growth factor 1 enhances the migratory capacity of mesenchymal stem cells." Biochem Biophys Res Commun **356**(3): 780-784.

Liechty, K., T. C. Mackenzie, A. Shaaban, A. Radu, A. B. Moseley, R. Deans, D. Marshak and A. Flake (2000). "Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep." Nat. Med. **6**(11): 1282-1286.

Linero, I. and O. Chaparro (2014). "Paracrine Effect of Mesenchymal Stem Cells Derived from Human Adipose Tissue in Bone Regeneration." PLoS One **9**(9): e107001.

Liu, Y., Y. Zheng, G. Ding, D. Fang, C. Zhang, P. M. Bartold, S. Gronthos, S. Shi and S. Wang (2008). "Periodontal ligament stem cell-mediated treatment for periodontitis in miniature swine." Stem Cells **26**(4): 1065-1073.

Luo, Q., Q. Kang, W. Si, W. Jiang, J. K. Park, Y. Peng, X. Li, H. H. Luu, J. Luo, A. G. Montag, R. C. Haydon and T. C. He (2004). "Connective tissue growth factor (CTGF) is regulated by Wnt and bone morphogenetic proteins signaling in osteoblast differentiation of mesenchymal stem cells." J Biol Chem **279**(53): 55958-55968.

Ma, D., L. Cui, J. Gao, W. Yan, Y. Liu, S. Xu and B. Wu (2014). "Proteomic Analysis of Mesenchymal Stem Cells from Normal and Deep Carious Dental Pulp." PLOS ONE **9**(5): e97026.

Ma, T., W. Grayson, M. Frohlich and G. Vunjak-Novakovic (2009). Hypoxia and Stem Cell-Based Engineering of Mesenchymal Tissues. Biotechnol. Prog. **25:** 32-42.

Ma, T., W. L. Grayson, M. Frohlich and G. Vunjak-Novakovic (2009). "Hypoxia and stem cell-based engineering of mesenchymal tissues." Biotechnol Prog **25**(1): 32-42.

Mackensen, A., R. Dräger, M. Schlesier, R. Mertelsmann and A. Lindemann (2000). "Presence of IgE antibodies to bovine serum albumin in a patient developing anaphylaxis after vaccination with human peptide-pulsed dendritic cells." Cancer Immunology, Immunotherapy **49**(3): 152-156.

Maffioli, E., S. Nonnis, R. Angioni, F. Santagata, B. Cali, L. Zanotti, A. Negri, A. Viola and G. Tedeschi (2017). "Proteomic analysis of the secretome of human bone marrow-derived mesenchymal stem cells primed by pro-inflammatory cytokines." J Proteomics **166**: 115-126.

Marx, R. E., E. R. Carlson, R. M. Eichstaedt, S. R. Schimmele, J. E. Strauss and K. R. Georgeff (1998). "Platelet-rich plasma: Growth factor enhancement for bone grafts." Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology **85**(6): 638-646.

Maurer, P., C. Hohenadl, E. Hohenester, W. Gohring, R. Timpl and J. Engel (1995). "The C-terminal portion of BM-40 (SPARC/osteonectin) is an autonomously folding and crystallisable domain that binds calcium and collagen IV." J Mol Biol **253**(2): 347-357.

Mead, B., A. Logan, M. Berry, W. Leadbeater and B. A. Scheven (2014). "Paracrine-mediated neuroprotection and neuritogenesis of axotomised retinal ganglion cells by human dental pulp stem cells: Comparison with human bone marrow and adipose-derived mesenchymal stem cells." PLoS ONE **9**(10).

Melcher, A. H. (1970). "Repair of wounds in the periodontium of the rat. Influence of periodontal ligament on osteogenesis." Archives of Oral Biology **15**(12).

Melin, M., A. Joffre-Romeas, J. C. Farges, M. L. Couble, H. Magloire and F. Bleicher (2000). "Effects of TGFbeta1 on dental pulp cells in cultured human tooth slices." Journal of dental research **79**(9): 1689.

Metsalu, T. and J. Vilo (2015). "ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap." Nucleic Acids Research **43**(Web Server issue): W566-W570.

Mi, H., A. Muruganujan, J. T. Casagrande and P. D. Thomas (2013). "Large-scale gene function analysis with the PANTHER classification system." Nature Protocols **8**: 1551.

Mita, T., Y. Furukawa-Hibi, H. Takeuchi, H. Hattori, K. Yamada, H. Hibi, M. Ueda and A. Yamamoto (2015). "Conditioned medium from the stem cells of human dental pulp improves cognitive function in a mouse model of Alzheimer's disease." Behavioural Brain Research **293**: 189-197.

Miura, M., S. Gronthos, M. Zhao, B. Lu, L. W. Fisher, P. G. Robey and S. Shi (2003). "SHED: Stem cells from human exfoliated deciduous teeth." Proceedings of the National Academy of Sciences **100**(10): 5807-5812.

Mohyeldin, A., T. Garzon-Muvdi and A. Quinones-Hinojosa (2010). "Oxygen in stem cell biology: a critical component of the stem cell niche." Cell Stem Cell **7**(2): 150-161.

Monnouchi, S., H. Maeda, A. Yuda, S. Hamano, N. Wada, A. Tomokiyo, K. Koori, H. Sugii, S. Serita and A. Akamine (2015). "Mechanical induction of interleukin - 11 regulates osteoblastic/cementoblastic differentiation of human periodontal ligament stem/progenitor cells." Journal of Periodontal Research **50**(2): 231-239.

Morsczeck, C., W. Gotz, J. Schierholz, F. Zeilhofer, U. Kuhn, C. Mohl, C. Sippel and K. H. Hoffmann (2005). "Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth." Matrix Biol **24**(2): 155-165.

Mrozik, K. M., N. Wada, V. Marino, W. Richter, S. Shi, D. L. Wheeler, S. Gronthos and P. M. Bartold (2013). "Regeneration of periodontal tissues using allogeneic periodontal ligament stem cells in an ovine model." Regen Med **8**(6): 711-723.

Mrozik, K. M., P. S. Zilm, C. J. Bagley, S. Hack, P. Hoffmann, S. Gronthos and P. M. Bartold (2010). "Proteomic Characterization of Mesenchymal Stem Cell-Like Populations Derived from Ovine Periodontal Ligament, Dental Pulp, and Bone Marrow: Analysis of Differentially Expressed Proteins." Stem Cells and Development **19**(10): 1485-1499.

Munir, H., L. S. C. Ward, L. Sheriff, S. Kemble, S. Nayar, F. Barone, G. B. Nash and H. M. McGettrick (2017). "Adipogenic Differentiation of Mesenchymal Stem Cells Alters Their Immunomodulatory Properties in a Tissue-Specific Manner." Stem Cells **35**(6): 1636-1646.

Nagata, M., K. Iwasaki, K. Akazawa, M. Komaki, N. Yokoyama, Y. Izumi and I. Morita (2017). "Conditioned Medium from Periodontal Ligament Stem Cells Enhances Periodontal Regeneration." Tissue engineering. Part A **23**(9-10): 367-377.

Nayak, T. R., L. Jian, L. C. Phua, H. K. Ho, Y. Ren and G. Pastorin (2010). "Thin films of functionalized multiwalled carbon nanotubes as suitable scaffold materials for stem cells proliferation and bone formation." ACS Nano **4**(12): 7717-7725.

Neufeld, G., T. Cohen, S. Gengrinovitch and Z. Poltorak (1999). "Vascular endothelial growth factor (VEGF) and its receptors." The FASEB Journal **13**(1): 9-22.

Nuñez, J., N. Sanchez, F. Vignoletti, I. Sanz-Martin, R. Caffesse, S. Santamaria, J. Garcia-Sanz and M. Sanz (2018). "Cell therapy with allogenic canine periodontal ligament-derived cells in periodontal regeneration of critical size defects." Journal of Clinical Periodontology **45**(4): 453-461.

Nuñez, J., S. Sanz‐Blasco, F. Vignoletti, F. Muñoz, H. Arzate, C. Villalobos, L. Nuñez, R. G. Caffesse and M. Sanz (2012). "Periodontal regeneration following implantation of cementum and periodontal ligament‐derived cells." Journal of Periodontal Research **47**(1): 33-44.

Nyman, S., J. Gottlow, J. Lindhe, T. Karring and J. Wennstrom (1987). "New attachment formation by guided tissue regeneration." Journal of Periodontal Research **22**(3): 252-254.

Ogata, K., W. Katagiri, M. Osugi, T. Kawai, Y. Sugimura, H. Hibi, S. Nakamura and M. Ueda (2015). "Evaluation of the therapeutic effects of conditioned media from mesenchymal stem cells in a rat bisphosphonate-related osteonecrosis of the jaw-like model." Bone **74**: 95-105.

Ogata, K., M. Matsumura, M. Moriyama, W. Katagiri, H. Hibi and S. Nakamura (2018). "Cytokine Mixtures Mimicking Secretomes From Mesenchymal Stem Cells Improve Medication-Related Osteonecrosis of the Jaw in a Rat Model." JBMR Plus **2**(2): 69-80.

Ong, S.-E., B. Blagoev, I. Kratchmarova, D. B. Kristensen, H. Steen, A. Pandey and M. Mann (2002). "Stable Isotope Labeling by Amino Acids in Cell Culture, SILAC, as a Simple and Accurate Approach to Expression Proteomics." Molecular & Cellular Proteomics **1**(5): 376-386.

Orlowski, W. A. (1978). "Biochemical study of collagen turnover in rat incisor periodontal ligament." Archives of Oral Biology **23**(12): 1163-1165.

Oskowitz, A., H. McFerrin, M. Gutschow, M. L. Carter and R. Pochampally (2011). "Serum-deprived human multipotent mesenchymal stromal cells (MSCs) are highly angiogenic." Stem Cell Research **6**(3): 215-225.

Osugi, M., W. Katagiri, R. Yoshimi, T. Inukai, H. Hibi and M. Ueda (2012). "Conditioned Media from Mesenchymal Stem Cells Enhanced Bone Regeneration in Rat Calvarial Bone Defects." Tissue Eng. Part A **18**(13-14): 1479-1489.

Palmer, R. M. and P. Cortellini (2008). "Periodontal tissue engineering and regeneration: Consensus report of the sixth european workshop on periodontology." J. Clin. Periodontol. **35**: 83-86.

Papapanou, P. N., M. Sanz, N. Buduneli, T. Dietrich, M. Feres, D. H. Fine, T. F. Flemmig, R. Garcia, W. V. Giannobile, F. Graziani, H. Greenwell, D. Herrera, R. T. Kao, M. Kebschull, D. F. Kinane, K. L. Kirkwood, T. Kocher, K. S. Kornman, P. S. Kumar, B. G. Loos, E. Machtei, H. Meng, A. Mombelli, I. Needleman, S. Offenbacher, G. J. Seymour, R. Teles and M. S. Tonetti (2018). "Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions." Journal of Clinical Periodontology **45**(S20): S162-S170.

Paradisi, M., F. Alviano, S. Pirondi, G. Lanzoni, M. Fernandez, G. Lizzo, L. Giardino, A. Giuliani, R. Costa, C. Marchionni, L. Bonsi and L. Calza (2014). "Human mesenchymal stem cells produce bioactive neurotrophic factors: source, individual variability and differentiation issues." Int J Immunopathol Pharmacol **27**(3): 391-402.

Park, J. Y., S. H. Jeon and P. H. Choung (2011). "Efficacy of periodontal stem cell transplantation in the treatment of advanced periodontitis." Cell Transplant **20**(2): 271-285.

Park, S.-J., H.-S. Bae and J.-C. Park (2015). "Osteogenic differentiation and gene expression profile of human dental follicle cells induced by human dental pulp cells." Journal of Molecular Histology **46**(1): 93-106.

Paschalidis, T., A. Bakopoulou, P. Papa, G. Leyhausen, W. Geurtsen and P. Koidis (2014). "Dental pulp stem cells' secretome enhances pulp repair processes and compensates TEGDMA-induced cytotoxicity." Dent. Mater. **30**(12): E405-E418.

Pauklin, S. and L. Vallier (2015). "Activin/Nodal signalling in stem cells." Development **142**(4): 607-619.

Pavasant, P. and T. Yongchaitrakul (2008). "Secreted protein acidic, rich in cysteine induces pulp cell migration via alphavbeta3 integrin and extracellular signal-regulated kinase." Oral Dis **14**(4): 335-340.

Pitt, J. J. (2009). "Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry." The Clinical biochemist. Reviews **30**(1): 19-34.

Popp, F., E. Eggenhofer, P. Renner, P. Slowik, S. A. Lang, H. Kaspar, E. Geissler, P. Piso, H. Schlitt and M. Dahlke (2008). "Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate." Transpl. Immunol. **20**(1-2): 55-60.

Qiu, J., X. Wang, H. Zhou, C. Zhang, Y. Wang, J. Huang, M. Liu, P. Yang and A. Song (2020). "Enhancement of periodontal tissue regeneration by conditioned media from gingiva-derived or periodontal ligament-derived mesenchymal stem cells: a comparative study in rats." Stem Cell Res Ther **11**(1): 42.

Quarles, L. D., D. A. Yohay, L. W. Lever, R. Caton and R. J. Wenstrup (1992). "Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development." J Bone Miner Res **7**(6): 683-692.

Rajan, T. S., S. Giacoppo, F. Diomede, P. Ballerini, M. Paolantonio, M. Marchisio, A. Piattelli, P. Bramanti, E. Mazzon and O. Trubiani (2016). "The secretome of periodontal ligament stem cells from MS patients protects against EAE." Scientific Reports **6**: 38743.

Ranganath, Sudhir H., O. Levy, Maneesha S. Inamdar and Jeffrey M. Karp (2012). "Harnessing the Mesenchymal Stem Cell Secretome for the Treatment of Cardiovascular Disease." Cell Stem Cell **10**(3): 244-258.

Ratajczak, J., P. Hilkens, P. Gervois, E. Wolfs, R. Jacobs, I. Lambrichts and A. Bronckaers (2016). "Angiogenic Capacity of Periodontal Ligament Stem Cells Pretreated with Deferoxamine and/or Fibroblast Growth Factor-2." PloS one **11**(12): e0167807-e0167807.

Reichert, T., S. Störkel, K. Becker and L. W. Fisher (1992). "The role of osteonectin in human tooth development: An immunohistological study." Calcified Tissue International **50**(5): 468-472.

Rippin, J. W. (1976). "Collagen turnover in the periodontal ligament under normal and altered functional forces." Journal of Periodontal Research **11**(2): 101-107.

Rodan, S. B., Y. Imai, M. A. Thiede, G. Wesolowski, D. Thompson, Z. Bar-Shavit, S. Shull, K. Mann and G. A. Rodan (1987). "Characterization of a human osteosarcoma cell line (Saos-2) with osteoblastic properties." Cancer Res **47**(18): 4961-4966.

Rosset, E. M. and A. D. Bradshaw (2016). "SPARC/osteonectin in mineralized tissue." Matrix Biology **52-54**: 78-87.

Rutkovskiy, A., K.-O. Stensløkken and I. J. Vaage (2016). "Osteoblast Differentiation at a Glance." Medical science monitor basic research **22**: 95-106.

Sakaguchi, K., W. Katagiri, M. Osugi, T. Kawai, Y. Sugimura-Wakayama and H. Hibi (2017). "Periodontal tissue regeneration using the cytokine cocktail mimicking secretomes in the conditioned media from human mesenchymal stem cells." Biochem Biophys Res Commun **484**(1): 100-106.

Sakdee, J. B., R. R. White, T. C. Pagonis and P. V. Hauschka (2009). "Hypoxia-amplified Proliferation of Human Dental Pulp Cells." Journal of Endodontics **35**(6): 818-823.

Sanchez, C., A. Oskowitz and R. R. Pochampally (2009). "Epigenetic Reprogramming of IGF1 and Leptin Genes by Serum Deprivation in Multipotential Mesenchymal Stromal Cells." STEM CELLS **27**(2): 375-382.

Sarojini, H., R. Estrada, H. Lu, S. Dekova, M. J. Lee, R. D. Gray and E. Wang (2008). "PEDF from mouse mesenchymal stem cell secretome attracts fibroblasts." J Cell Biochem **104**(5): 1793-1802.

Scheven, B., J. Man, J. Millard, P. Cooper, S. Lea, A. Walmsley and A. Smith (2009). "VEGF and odontoblast-like cells: stimulation by low frequency ultrasound." Archieves of Oral Biology **54**: 185-191.

Schüpbach, P., T. Gaberthüel, F. Lutz and B. Guggenheim (1993). "Periodontal repair or regeneration: structures of different types of new attachment." Journal of Periodontal Research **28**(4): 281-293.

Seo, B.-M., M. Miura, S. Batouli, M. Young, P. G. Robey, S. Shi, J. Brahim, S. Gronthos, P. M. Bartold and C.-Y. Wang (2004). "Investigation of multipotent postnatal stem cells from human periodontal ligament." Lancet **364**(9429): 149-155.

Shi, Y., Z. Yuming, M. Yushi and G. Lihong (2016). "Profiling the Secretome of Human Stem Cells from Dental Apical Papilla." Stem Cells and Development **25**(6): 499-508.

Shiba, H., Y. Uchida, K. Kamihagi, M. Sakata, T. Fujita, S. Nakamura, T. Takemoto, Y. Kato and H. Kurihara (2001). "Transforming growth factor-beta1 and basic fibroblast growth factor modulate osteocalcin and osteonectin/SPARC syntheses in vitamin-D-activated pulp cells." J Dent Res **80**(7): 1653-1659.

Shigematsu, S., K. Yamauchi, K. Nakajima, S. Iijima, T. Aizawa and K. Hashizume (1999). "IGF-1 regulates migration and angiogenesis of human endothelial cells." Endocr J **46 Suppl**: S59-62.

Skougaard, M. R., B. M. Levy and J. Simpson (1970). "Collagen metabolism in skin and periodontal membrane of the marmoset." European Journal of Oral Sciences **78**(1 - 4): 256-262.

Smith, J. G. W. (2012). Cellular responses to dental extracellular matrix molecules / by James George William Smith.

Sonoyama, W., Y. Liu, T. Yamaza, R. S. Tuan, S. Wang, S. Shi and G. T. J. Huang (2008). "Characterization of the Apical Papilla and Its Residing Stem Cells from Human Immature Permanent Teeth: A Pilot Study." Journal of Endodontics **34**(2): 166-171.

Spahr, L., Y. Chalandon, S. Terraz, V. Kindler, L. Rubbia-Brandt, J.-L. Frossard, R. Breguet, N. Lanthier, A. Farina, J. Passweg, C. Becker and A. Hadengue (2013). "Autologous Bone Marrow Mononuclear Cell Transplantation in Patients with Decompensated Alcoholic Liver Disease: A Randomized Controlled Trial." PLoS One **8**(1).

Spencer, J. A., F. Ferraro, E. Roussakis, A. Klein, J. Wu, J. M. Runnels, W. Zaher, L. J. Mortensen, C. Alt, R. Turcotte, R. Yusuf, D. Côté, S. A. Vinogradov, D. T. Scadden and C. P. Lin (2014). "Direct measurement of local oxygen concentration in the bone marrow of live animals." Nature **508**(7495): 269-273.

Stanley, E. R. (1998). Macrophage Colony-Stimulating Factor (CSF-1). Encyclopedia of Immunology (Second Edition). P. J. Delves. Oxford, Elsevier**:** 1650-1654.

Suaid, F. F., F. V. Ribeiro, T. R. L. E. S. Gomes, K. G. Silvério, M. D. Carvalho, F. H. Nociti, M. Z. Casati and E. A. Sallum (2012). "Autologous periodontal ligament cells in the treatment of class III furcation defects: a study in dogs." Journal of Clinical Periodontology **39**(4): 377-384.

Tachida, Y., H. Sakurai, J. Okutsu, K. Suda, R. Sugita, Y. Yaginuma, Y. Ogura, K. Shimada, F. Isono, K. Kubota and H. Kobayashi (2015). "Proteomic Comparison of the Secreted Factors of Mesenchymal Stem Cells from Bone Marrow, Adipose Tissue and Dental Pulp." Journal of Proteomics & Bioinformatics **8**(12): 266-266.

Takahashi, H. and M. Shibuya (2005). "The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions." Clinical Science **109**(3): 227-241.

Takahashi, K. and S. Yamanaka (2006). "Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors." Cell **126**(4): 663-676.

Tasso, R., M. Gaetani, E. Molino, A. Cattaneo, M. Monticone, A. Bachi and R. Cancedda (2012). "The role of bFGF on the ability of MSC to activate endogenous regenerative mechanisms in an ectopic bone formation model." Biomaterials **33**(7): 2086-2096.

Tetsuro, W. and M. Kohei (2009). "Roles of TGF-β family signaling in stem cell renewal and differentiation." Cell Research **19**(1): 103.

Thomas, P. D., M. J. Campbell, A. Kejariwal, H. Mi, B. Karlak, R. Daverman, K. Diemer, A. Muruganujan and A. Narechania (2003). "PANTHER: a library of protein families and subfamilies indexed by function." Genome Res **13**(9): 2129-2141.

Thompson, A., J. Schäfer, K. Kuhn, S. Kienle, J. Schwarz, G. Schmidt, T. Neumann and C. Hamon (2003). "Tandem Mass Tags: A Novel Quantification Strategy for Comparative Analysis of Complex Protein Mixtures by MS/MS." Analytical Chemistry **75**(8): 1895-1904.

Timmers, L., S. K. Lim, I. E. Hoefer, F. Arslan, R. C. Lai, A. A. van Oorschot, M. J. Goumans, C. Strijder, S. K. Sze, A. Choo, J. J. Piek, P. A. Doevendans, G. Pasterkamp and D. P. de Kleijn (2011). "Human mesenchymal stem cell-conditioned medium improves cardiac function following myocardial infarction." Stem Cell Res **6**(3): 206-214.

Torii, D., K. Konishi, N. Watanabe, S. Goto and T. Tsutsui (2015). "Cementogenic potential of multipotential mesenchymal stem cells purified from the human periodontal ligament." Official Journal of the Society of the Nippon Dental University **103**(1): 27-35.

Trombetta, J. M., A. D. Bradshaw and R. H. Johnson (2010). "SPARC/Osteonectin Functions to Maintain Homeostasis of the Collagenous Extracellular Matrix in the Periodontal Ligament." Journal of Histochemistry & Cytochemistry **58**(10): 871-879.

Tsuchiya, S., K. Hara, M. Ikeno, Y. Okamoto, H. Hibi and M. Ueda (2013). "Rat bone marrow stromal cell-conditioned medium promotes early osseointegration of titanium implants." The International journal of oral & maxillofacial implants **28**(5): 1360-1369.

Tuncay, O. C., J. C. Haselgrove, P. Frasca, C. Piddington and I. M. Shapiro (1990). "Scanning microfluorimetric measurements of redox status in the rat dento-alveolar tissues." Archives of Oral Biology **35**(2): 113-118.

Ukaji, T., Y. Lin, K. Banno, S. Okada and K. Umezawa (2015). "Inhibition of IGF-1-Mediated Cellular Migration and Invasion by Migracin A in Ovarian Clear Cell Carcinoma Cells." PloS one **10**(9): e0137663-e0137663.

Väänänen, K. (2005). "Mechanism of osteoclast mediated bone resorption - Rationale for the design of new therapeutics." Advanced Drug Delivery Reviews **57**(7): 959-971.

Vater, C., P. Kasten and M. Stiehler (2011). "Culture media for the differentiation of mesenchymal stromal cells." Acta Biomaterialia **7**(2): 463-477.

Volmer, M. W., K. Stühler, M. Zapatka, A. Schöneck, S. Klein‐Scory, W. Schmiegel, H. E. Meyer and I. Schwarte‐Waldhoff (2005). "Differential proteome analysis of conditioned media to detect Smad4 regulated secreted biomarkers in colon cancer." PROTEOMICS **5**(10): 2587-2601.

Wakayama, H., N. Hashimoto, Y. Matsushita, K. Matsubara, N. Yamamoto, Y. Hasegawa, M. Ueda and A. Yamamoto (2015). "Factors secreted from dental pulp stem cells show multifaceted benefits for treating acute lung injury in mice." Cytotherapy **17**(8): 1119-1129.

Wang, F., H. Zhou, Z. Du, X. Chen, F. Zhu, Z. Wang, Y. Zhang, L. Lin, M. Qian, X. Zhang, X. Li and A. Hao (2015). "Cytoprotective effect of melatonin against hypoxia/serum deprivation-induced cell death of bone marrow mesenchymal stem cells in vitro." European Journal of Pharmacology **748**: 157-165.

Wang, K. X., L. L. Xu, Y. F. Rui, S. Huang, S. E. Lin, J. H. Xiong, Y. H. Li, W. Y. Lee and G. Li (2015). "The effects of secretion factors from umbilical cord derived mesenchymal stem cells on osteogenic differentiation of mesenchymal stem cells." PLoS One **10**(3): e0120593.

Wang, S., X. Qu and R. C. Zhao (2012). "Clinical applications of mesenchymal stem cells." Journal of Hematology and Oncology **5**: 19.

Watabe, T. and K. Miyazono (2009). "Roles of TGF-beta family signaling in stem cell renewal and differentiation." Cell Res **19**(1): 103-115.

Wu, Y., L. Chen, P. G. Scott and E. E. Tredget (2007). "Mesenchymal Stem Cells Enhance Wound Healing Through Differentiation and Angiogenesis." STEM CELLS **25**(10): 2648-2659.

Wu, Y., Y. Yang, P. Yang, Y. Gu, Z. Zhao, L. Tan, L. Zhao, T. Tang and Y. Li (2013). "The osteogenic differentiation of PDLSCs is mediated through MEK/ERK and p38 MAPK signalling under hypoxia." Archives of Oral Biology **58**(10): 1357-1368.

Xia, Y., H.-N. Tang, R.-X. Wu, Y. Yu, L.-N. Gao and F.-M. Chen (2016). "Cell Responses to Conditioned Media Produced by Patient-Matched Stem Cells Derived From Healthy and Inflamed Periodontal Ligament Tissues." Journal of Periodontology **87**(5): e53-e63.

Xinaris, C., M. Morigi, V. Benedetti, B. Imberti, A. S. Fabricio, E. Squarcina, A. Benigni, E. Gagliardini and G. Remuzzi (2013). "A novel strategy to enhance mesenchymal stem cell migration capacity and promote tissue repair in an injury specific fashion." Cell Transplant **22**(3): 423-436.

Xu, J., B. Wang, Y. Sun, T. Wu, Y. Liu, J. Zhang, W. Y. Lee, X. Pan, Y. Chai and G. Li (2016). "Human fetal mesenchymal stem cell secretome enhances bone consolidation in distraction osteogenesis." Stem Cell Research & Therapy **7**(1).

Yalvaç, M. E., A. Yarat, D. Mercan, A. A. Rizvanov, A. Palotás and F. Şahin (2013). "Characterization of the secretome of human tooth germ stem cells (hTGSCs) reveals neuro-protection by fine-tuning micro-environment." Brain, Behavior, and Immunity **32**(0): 122-130.

Yamada, Y., M. Ueda, H. Hibi and S. Baba (2006). "A novel approach to periodontal tissue regeneration with mesenchymal stem cells and platelet-rich plasma using tissue engineering technology: A clinical case report." Int J Periodontics Restorative Dent **26**(4): 363-369.

Yamaguchi, S., R. Shibata, N. Yamamoto, M. Nishikawa, H. Hibi, T. Tanigawa, M. Ueda, T. Murohara and A. Yamamoto (2015). "Dental pulp-derived stem cell conditioned medium reduces cardiac injury following ischemia-reperfusion." Scientific reports **5**: 16295.

Yamane, T., T. Kunisada, H. Yamazaki, T. Era, T. Nakano and S.-I. Hayashi (1997). "Development of Osteoclasts From Embryonic Stem Cells Through a Pathway That Is c-fms but not c-kit Dependent." Blood **90**(9): 3516-3523.

Yang, Y., F. M. Rossi and E. E. Putnins (2010). "Periodontal regeneration using engineered bone marrow mesenchymal stromal cells." Biomaterials **31**(33): 8574-8582.

Yoo, P. S., A. L. Mulkeen and C. H. Cha (2006). "Post-transcriptional regulation of vascular endothelial growth factor: Implications for tumor angiogenesis." World Journal of Gastroenterology : WJG **12**(31): 4937-4942.

Yourek, G., M. A. Hussain and J. J. Mao (2007). "Cytoskeletal changes of mesenchymal stem cells during differentiation." Asaio j **53**(2): 219-228.

Youssef, A., D. Aboalola and V. K. M. Han (2017). "The Roles of Insulin-Like Growth Factors in Mesenchymal Stem Cell Niche." Stem Cells International **2017**: 9453108.

Youssef, A., D. Aboalola and V. K. M. Han (2017). "The Roles of Insulin-Like Growth Factors in Mesenchymal Stem Cell Niche." Stem Cells International **2017**: 12.

Yu, C. Y., N. M. Boyd, S. J. Cringle, V. A. Alder and D. Y. Yu (2002). "Oxygen distribution and consumption in rat lower incisor pulp." Archives of Oral Biology **47**(7): 529-536.

Yu, J., H. He, C. Tang, G. Zhang, Y. Li, R. Wang, J. Shi and Y. Jin (2010). "Differentiation potential of STRO-1(+)dental pulp stem cells changes during cell passaging." BMC Cell Biology **11**: 32-32.

Zhang, F., T. Ren, J. Wu and J. Niu (2015). "Small concentrations of TGF-beta1 promote proliferation of bone marrow-derived mesenchymal stem cells via activation of Wnt/beta-catenin pathway." Indian J Exp Biol **53**(8): 508-513.

Zhang, L. and J. E. Elias (2017). "Relative Protein Quantification Using Tandem Mass Tag Mass Spectrometry." Methods Mol Biol **1550**: 185-198.

Zhang, Q., S. Shi, Y. Liu, J. Uyanne, Y. Shi, S. Shi and A. D. Le (2009). "Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis." J Immunol **183**(12): 7787-7798.

Zhang, Q. B., Z. Q. Zhang, S. L. Fang, Y. R. Liu, G. Jiang and K. F. Li (2014). "Effects of hypoxia on proliferation and osteogenic differentiation of periodontal ligament stem cells: an in vitro and in vivo study." Genet Mol Res **13**(4): 10204-10214.

Zhou, Y., W. Fan and Y. Xiao (2014). "The Effect of Hypoxia on the Stemness and Differentiation Capacity of PDLC and DPC." BioMed Research International **2014**.

Zhou, Y., W. Fan and Y. Xiao (2014). "The Effect of Hypoxia on the Stemness and Differentiation Capacity of PDLC and DPC." BioMed Research International **2014**: 7.

9 APPENDIX

9.1 Appendix 1

LC-MS/MS Experiment

UltiMate® 3000 HPLC series (Dionex, Sunnyvale, CA USA) is used for peptide concentration and separation. Samples are trapped on uPrecolumn Cartridge, Acclaim PepMap 100 C18, 5 um, 100A 300um i.d. x 5mm (Dionex, Sunnyvale, CA USA) and separated in Nano Series™ Standard Columns 75 µm i.d. x 15 cm, packed with C18 PepMap100, 3 µm, 100Å (Dionex, Sunnyvale, CA USA). The gradient used is from 3.2% to 24% solvent B (0.1% formic acid in acetonitrile) for 30 mins and then increase to 60% in 15 min. The column is washed with 80% solvent B for 15mins and equilibrate with 3.2% solvent B for another 15 mins. The total run time is 75 mins. Peptides were eluted directly (~ 350 nL min^{-1}) via a Triversa Nanomate nanospray source (Advion Biosciences, NY) into a QExactive HF (QEHF) mass spectrometer (Thermo Fisher Scientific). The data-dependent scanning acquisition is controlled by Xcalibur 4.0 software. The mass spectrometer alternated between a full FT-MS scan (m/z 375 – 1600) and subsequent high energy collision dissociation (HCD) MS/MS scans of the 20 most abundant ions. Survey scans are acquired in the QEHF with a resolution of 120 000 at m/z 200 and automatic gain control (AGC) $3x10^6$. Precursor ions were fragmented in HCD MS/MS with resolution set up at 60,000 and a normalized collision energy of 32. ACG target for HCD MS/MS was $1x10^5$. The width of the precursor isolation window was 1.2 m/z and only multiply-charged precursor ions were selected for MS/MS. Spectra were acquired for 60 mins with dynamic exclusion time of 20s.

The MS and MS/MS scans are searched against Uniprot database using Proteome Discoverer 2.2 (ThermoFisher Scientific) with a 1% false discovery rate (FDR) criteria. Oxidation (M) and N terminal acetylation are set as variable modifications. Cysteine carbamidomethylation and TMT 6-plex on lysine and N-terminal as fixed modifications. The precursor mass tolerance is 10 ppm and the MS/MS mass tolerance was 0.02Da. The Quan method was set for reporter ions quantification with HCD and MS2. For the reporter ion abundance, only unique peptides were considered for the quantification.

9.2 Appendix 2

Proteins detected in BMSCs, PDLSCs and DPSCs via mass spectrometry

Accession Numbers	BMSCs	Accession Numbers	PDLSCs	Accession Numbers	DPSCs
P33436	72 kDa type IV collagenase	**Q7TP84**	Ab1-346	**P33436**	72 kDa type IV collagenase
Q7TP84	Ab1-346	**Q6GMN8**	Actn1 protein	**Q7TP84**	Ab1-346
A0A0G2K4M6	Actin, aortic smooth muscle	**Q80ZA3**	Alpha-2 antiplasmin	**Q6GMN8**	Actn1 protein
Q6GMN8	Actn1 protein	**P06238**	Alpha-2-macroglobulin	**A0A0H2UHL3**	Adipocyte enhancer-binding protein 1
A0A0H2UHL3	Adipocyte enhancer-binding protein 1	**A0A0G2K013**	Alpha-actinin-4	**Q63041**	Alpha-1-macroglobulin
Q63041	Alpha-1-macroglobulin	**P07151**	Beta-2-microglobulin	**P24090**	Alpha-2-HS-glycoprotein
Q80ZA3	Alpha-2 antiplasmin	**P47853**	Biglycan	**A0A0G2K013**	Alpha-actinin-4
P24090	Alpha-2-HS-glycoprotein	**P18418**	Calreticulin	**A0A0G2K8F6**	Alpha-mannosidase
A0A0G2K013	Alpha-actinin-4	**P15087**	Carboxypeptidase E	**Q6P6Q5**	Amyloid beta A4 protein
P07150	Annexin A1	**P00787**	Cathepsin B	**Q99J86**	Attractin
P07151	Beta-2-microglobulin	**P07154**	Cathepsin L1	**P07151**	Beta-2-microglobulin
P47853	Biglycan	**A0A0G2KAN1**	Collagen alpha-2(I) chain	**P47853**	Biglycan
Q6Q0N0	Calsyntenin-1	**P02454**	Collagen alpha-1(I) chain	**Q4G030**	C1r protein (Fragment)
A0A0G2K4K3	Canalicular multispecific organic anion transporter 2	**P13941**	Collagen alpha-1(III) chain	**Q6Q0N0**	Calsyntenin-1
P15087	Carboxypeptidase E	**P20909**	Collagen alpha-1(XI) chain	**P00787**	Cathepsin B
Q6IRK9	Carboxypeptidase Q	**M0RBJ7**	Complement C3	**P07154**	Cathepsin L1
A0A0G2JXN9	Cartilage oligomeric matrix protein	**Q6IN11**	Connective tissue growth factor	**P02454**	Collagen alpha-1(I) chain
P00787	Cathepsin B	**P14841**	Cystatin-C	**F1LRM7**	Collagen alpha-1(II) chain
P07154	Cathepsin L1	**Q8CHN5**	Epididymal secretory protein 1	**P13941**	Collagen alpha-1(III) chain
P02454	Collagen alpha-1(I) chain	**F1LST1**	Fibronectin	**G3V763**	Collagen alpha-1(V) chain
P13941	Collagen alpha-1(III) chain	**G3V6X1**	Fibulin 2, isoform CRA_a	**P20909**	Collagen alpha-1(XI) chain
G3V763	Collagen alpha-1(V) chain	**D3ZQ25**	Fibulin-1	**A0A0G2KAN1**	Collagen alpha-2(I) chain
P20909	Collagen alpha-1(XI) chain	**Q9WVH8**	Fibulin-5	**M0RBJ7**	Complement C3

A0A0G2KAJ7	Collagen alpha-1(XII) chain		**C0JPT7**	Filamin alpha		**A0A096P6L9**	Complement C5
A0A0G2KAN1	Collagen alpha-2(I) chain		**Q62632**	Follistatin-related protein 1		**Q6IN11**	Connective tissue growth factor
M0RBJ7	Complement C3		**Q64599**	Hemiferrin		**Q4TU93**	C-type mannose receptor 2
Q6IN11	Connective tissue growth factor		**F1M9B2**	Insulin-like growth factor binding protein 7, isoform CRA_b		**P14841**	Cystatin-C
P14841	Cystatin-C		**P12843**	Insulin-like growth factor-binding protein 2		**Q01129**	Decorin
Q08420	Extracellular superoxide dismutase [Cu-Zn]		**P70490**	Lactadherin		**D3Z9E1**	Elastin microfibril interfacer 1 (Predicted), isoform CRA_b
G3V9M6	Fibrillin 1, isoform CRA_a		**O35806**	Latent-transforming growth factor beta-binding protein 2		**F1LQ45**	Epsin-2
P04937	Fibronectin		**P51886**	Lumican		**Q08420**	Extracellular superoxide dismutase [Cu-Zn]
G3V6X1	Fibulin 2, isoform CRA_a		**P30121**	Metalloproteinase inhibitor 2		**G3V9M6**	Fibrillin 1, isoform CRA_a
Q9WVH8	Fibulin-5		**F1LM84**	Nidogen-1		**F1LST1**	Fibronectin
C0JPT7	Filamin alpha		**Q63083**	Nucleobindin-1		**A0A096P6L8**	Fibronectin
Q62632	Follistatin-related protein 1		**A0A097BW25**	Periostin isoform R1		**G3V6X1**	Fibulin 2, isoform CRA_a
Q68FP1	Gelsolin		**O08628**	Procollagen C-endopeptidase enhancer 1		**D3ZQ25**	Fibulin-1
Q642B0	Glypican 4		**F1LNH3**	Procollagen, type VI, alpha 2, isoform CRA_a		**Q9WVH8**	Fibulin-5
Q64599	Hemiferrin		**D4A619**	Protein Cntrl		**C0JPT7**	Filamin alpha
Q5RJM3	Insulin-like growth factor binding protein 7		**F1LQ00**	Protein Col5a2		**Q5U2V1**	FK506 binding protein 10
P12843	Insulin-like growth factor-binding protein 2		**D3ZUL3**	Protein Col6a1		**Q62632**	Follistatin-related protein 1
Q5PQQ8	Integrin beta-like protein 1		**D3ZFH5**	Protein Itih2		**Q63772**	Growth arrest-specific protein 6
D3ZBS2	Inter-alpha-trypsin inhibitor heavy chain H3		**A0A0G2KAH2**	Protein Pxdn		**Q64599**	Hemiferrin
Q62611	Interleukin-1 receptor-like 1		**P05964**	Protein S100-A6		**F1M9B2**	Insulin-like growth factor binding protein 7, isoform CRA_b

P70490	Lactadherin		**Q5M7T5**	Protein Serpinc1		**P12843**	Insulin-like growth factor-binding protein 2
O35806	Latent-transforming growth factor beta-binding protein 2		**P11980**	Pyruvate kinase PKM		**A0A0G2JVW1**	Insulin-like growth factor-binding protein 5
Q64361	Latexin		**Q5XI73**	Rho GDP-dissociation inhibitor 1		**D3ZBS2**	Inter-alpha-trypsin inhibitor heavy chain H3
D3ZP82	Lysyl oxidase-like 3 (Predi		**P16975**	SPARC		**Q6IFV1**	Keratin, type I cytoskeletal 14
D3Z8U5	Metalloendopeptidase		**Q71SA3**	Thrombospondin 1		**Q5XI51**	Kinesin-like protein KIF2B
P30120	Metalloproteinase inhibitor 1		**Q91ZP7**	Tissue inhibitor of metalloproteinase 1 (Fragment)		**M0R6K0**	Laminin subunit beta-2
W4VSR4	Nidogen-2		**P31232**	Transgelin		**D3ZAA3**	Latent transforming growth factor beta binding protein 1
Q63083	Nucleobindin-1		**A0A0G2K2G8**	Tropomyosin alpha-4 chain		**O35806**	Latent-transforming growth factor beta-binding protein 2
D3ZVB7	Osteoglycin (Predicted)		**G3V8C3**	Vimentin		**P51886**	Lumican
D3ZAF5	Periostin, osteoblast specific factor		**P85972**	Vinculin		**A0A096MK30**	Moesin
P20961	Plasminogen activator inhibitor 1					**F1M0Z6**	Neogenin
Q5RJP7	Platelet-derived growth factor receptor-like protein					**F1LM84**	Nidogen-1
B5DEY0	Pls1 protein					**B5DFC9**	Nidogen-2
P0CG51	Polyubiquitin-B					**A0A097BW25**	Periostin isoform R1
O08628	Procollagen C-endopeptidase enhancer 1					**Q9Z135**	Procollagen C-proteinase 3 (Fragment)
F1LNH3	Procollagen, type VI, alpha 2, isoform CRA_a					**F1LNH3**	Procollagen, type VI, alpha 2, isoform CRA_a
P62963	Profilin-1					**Q6P7A4**	Prosaposin
F1MA59	Protein Col4a1					**F1M305**	Protein Abi3bp
F1M6Q3	Protein Col4a2					**A0A0G2K3A9**	Protein Adamts2
F1LQ00	Protein Col5a2					**F1MA59**	Protein Col4a1
D3ZUL3	Protein Col6a1					**F1M6Q3**	Protein Col4a2
A0A0G2KAI7	Protein Efemp2					**F1LQ00**	Protein Col5a2
Q62902	Protein ERGIC-53					**D3ZUL3**	Protein Col6a1
D3ZFH5	Protein Itih2					**D3ZFH5**	Protein Itih2

D3ZQN7	Protein Lamb1					**D3ZQN7**	Protein Lamb1
F1MAA7	Protein Lamc1					**F1MAA7**	Protein Lamc1
Q58NB7	Protein Rarres1					**F1LRT0**	Protein Ltbp3
Q6B345	Protein S100-A11					**E9PSP1**	Protein Pltp
P05964	Protein S100-A6					**A0A0G2JWB6**	Protein Pxdn
D3ZAT4	Protein Serpina9					**D3ZAT4**	Protein Serpina9
Q5M7T5	Protein Serpinc1					**Q5M7T5**	Protein Serpinc1
D3ZSC1	Protein Susd5					**D3ZSC1**	Protein Susd5
D4A2G6	Protein Thbs2					**P16975**	SPARC
A0A0G2K1L0	Protein Tnc					**Q71SA3**	Thrombospondin 1
Q5XI73	Rho GDP-dissociation inhibitor 1					**Q91ZP7**	Tissue inhibitor of metalloproteinase 1 (Fragment)
Q9QZK5	Serine protease HTRA1					**P85972**	Vinculin
P16975	SPARC						
P0C6B8	Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1						
Q71SA3	Thrombospondin 1						
G3V6K1	Transcobalamin 2, isoform CRA_a						
P31232	Transgelin						
A0A0G2K2G8	Tropomyosin alpha-4 chain						
G3V8C3	Vimentin						
P85972	Vinculin						

9.3 Appendix 3

Proteins detected via TMT/Mass spectrometry

Accession	Description	MW [kDa]	BM 3N	BM 3H	PDL 3N	PDL 3H	DP 3N	DP 3H	MASTER
P02454	Collagen alpha-1(I) chain	137.9	109.2	83.9	160.5	142.45	86.95	71.25	101
P02466	Collagen alpha-2(I) chain	129.5	60.8	77	101.6	114.45	97.55	106.7	107.3
P04937	Fibronectin	272.3	95.3	116.1	74.3	68.1	86.1	119.2	89.4
P12346	Serotransferrin	76.3	168.8	171.1	62.3	61.1	51.9	54.2	73.2
P13941	Collagen alpha-1(III) chain	138.9	126.2	157.3	93.6	110.6	70.6	96.1	86.1
P16975	SPARC	34.3	66.05	91.65	84.4	94.8	134.35	115.95	102.4
P24594	Insulin-like growth factor-binding protein 5	30.3		26.25	12.55	12.95	290.2	244.95	111.3
P47853	Biglycan	41.7	111.1	105.7	45.2	49.5	114.5	155.3	96
P70566	Tropomodulin-2	39.5	290.25	176.05	57.45	52.95	18.95	56	81.2
Q62632	Follistatin-related protein 1	34.6	38.1	112.5	94	99.7	88.1	105	106.1
Q9R1E9	Connective tissue growth factor	37.7	121.4	115.9	43.6	63.2	103.2	128.6	88.3

9.4 Appendix 4

ABSTRACTS/ CONFERENCES

1. F.Ariffin, P.R Cooper, B.A Scheven. Effects Of Hypoxia And Serum Concentration On Dental- And Bone Marrow-Derived Mesenchymal Stem Cell Secretomes. College of Medical and Dental Sciences Festival of Graduate Research, 28th March 2017, University of Birmingham.

2. F.Ariffin, P.R Cooper, B.A Scheven. Comparison of Secretomes Derived from Periodontal Ligaments and Bone Marrow Mesenchymal Stem Cells. British Society for Oral and Dental Research (BSODR) Scientific Conference. 6-8th September 2017, Plymouth, United Kingdom.

3. Poster Prize award during Postgraduate Student Research Posters displayed at: A lecture by Professor Dame Amanda Fisher. Birmingham Dental School, University of Birmingham. 26th October 2017.

4. F.Ariffin, P.R Cooper, B.A Scheven. Effects of Hypoxic Conditions on Growth Factors in the Secretome from Periodontal Ligament, Bone Marrow and Dental Pulp Stem Cells. 2018 IADR/Pan European Regional Congress, 25-28th July 2018, London, England

5. F Ariffin; PR Cooper; MM Grant; BA Scheven. Growth Factor and Proteomic Profiles of Rat Periodontal Ligament Stem Cell Secretome. 13th Asia Pasific Society of Periodontology Conference Meeting. 28-29th September, Kuala Lumpur, Malaysia.