# Assessment of the Bioactive Potential of Demineralised Dentine Matrix on Bone Marrow-Derived, Mesenchymal Stem Cells

A Thesis Completed in Fulfilment of the Requirements for the Degree of Doctor of Philosophy (Ph.D.)



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#### **Thesis Summary**

At present, the prime candidate for augmentation of bone defects are autologous bone grafts (ABGs), owing to their ability to emulate the physiological signalling environment to induce bone marrow-derived, mesenchymal stem cells (BMMSCs) to differentiate into functional osteoblasts. However, the uses of ABGs are negated due to patient complications. Dentine matrix has recently become increasingly recognised as possessing bioactive nature and has demonstrated the ability to augment bone repair *in vivo*. However, the constituents of dentine that confer efficacy for bone formation are poorly understood. The aim of this study was to determine the potential for demineralised dentine matrix (DDM) to induce biological responses of BMMSCs and elucidate the key mediators required for bioactivity.

Isolation of a sub-population of BMMSCs yielded a population of cells with enhanced expansive capacity *in vitro*, with maintained mesenchymal stem cell marker expression. Application of DDM to BMMSCs significantly reduced cell expansion (0.1-10µg/mL), reduced apoptotic activity (10µg/mL) and enhanced migration (0.1µg/mL). Importantly, DDM at 10µg/mL directed osteogenesis of BMMSC by enhancing RunX2 gene expression after 5 days and deposition of a mineralised matrix after 28 days.

Western blot and enzyme-linked immunosorbent assay (ELISA) analyses of DDM identified a plethora of growth factors associated with directing osteogenesis of BMMSCs, including; transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ), bone morphogenetic protein-2 (BMP-2) and vascular epidermal growth factor (VEGF). Fractionation of DDM by heparin-affinity, to concentrate growth factors, resulted in diminished potential of DDM to enhance RunX2 expression and mineral deposition of BMMSCs. Depletion of decorin from DDM had no effect on osteogenic potential, however, depletion of biglycan attenuated RunX2 expression and mineral deposition. Substrates composed of silk-fibroin/gelatin (SF/G) at ratios of 75:25 supported attachment and expansion of BMMSCs with no discernible changes in morphology, compared to cells cultured on plastic. Addition of DDM to SF/G substrates resulted in enhanced BMMSC expansion (20µg/mL) and evidence of mineralised matrix deposition (5-20µg/mL), compared to un-loaded SF/G substrates.

In conclusion, DDM possesses bioactive potential towards BMMSCs, which is attributable to a range of synergistically acting growth factors along with additional matrix constituents. The ability of DDM to stimulate osteogenesis demonstrates potential for future developments in the field of tissue engineering of bone.

#### Abbreviations

α-ΜΕΜ	Minimum Essential Medium Eagle, Alpha Modifications
Ab	Antibody
ABG	Autologous Bone Graft
ABTS	2,2'-Azino-Bis(3-ethylbenzothiazoline-6-sulphonic acid)
AGE	Advanced Glycation End-product
Akt-1	RAC-alpha Serine/Threonine-Protein Kinase
AutoBT	Autologous Bone-Tooth graft
BAD	Bcl-2-Associated Death Promoter
BCA	Bicinchoninic Acid
BGN	Biglycan
BMD	Bone Mineral Density
BMMSC	Bone Marrow-Derived, Mesenchymal Stem Cell
ВМР	Bone Morphogenetic Protein
BMPR	Bone Morphogenetic Protein Receptor
ВР	Blocking Peptide
BSA	Bovine Serum Albumin
BSP	Bone Sialoprotein
BTE	Bone Tissue Engineering
CaCl <sub>2</sub>	Calcium Chloride
CaOH <sub>2</sub>	Calcium Hydroxide
Cbfa1	Core-Binding Factor Subunit Alpha (Alias: RunX2)
CCL2	Chemokine (C-C motif) Ligand 2
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CFU-F	Colony Forming Unit-Fibroblastic
CO <sub>2</sub>	Carbon Dioxide
COL1A1	Collagen Type I, alpha chain 1
CS	Chondroitin Sulphate

CTRL	Control
CXCR4 C-X-C	Chemokine Receptor Type 4
ddH <sub>2</sub> O	Deionised Distilled H <sub>2</sub> O
DDM	Demineralised Dentine Matrix
DEC	Decorin
Dlx5	Distal-Less Homeobox 5
DMP-1	Dentine Matrix Protein-1
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPP	Dentine Phosphoprotein
DPSC	Dental Pulp-Derived, Stem Cell
DS	Dermatan Sulphate
DSP	Dentine Sialoprotein
DSPP	Dentine Sialophosphoprotein
DSS	Disuccinimidyl Suberate
DTM	Demineralised Tooth Matrix
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetra-Acetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ESC	Embryonic Stem Cell
FACS	Fluorescence-Activated Cell Sorting
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FNA	Fibronectin-Adherent
FPLC	Fast-Performance Liquid Chromatography
FZD	Frizzled
G	Gelatin
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase

НАр	Hydroxyapatite
HCI	Hydrochloric Acid
HIF-1	Hypoxic Inducible Factor-1
HIV	Human Immunodeficiency Virus
HLA-DR	Human Leukocyte Antigen – Antigen D
HRP	Horseradish Peroxidase
IGF	Insulin-Like Growth Factor
IGFR	Insulin-Like Growth Factor Receptor
IL	Interleukin
IP	Immunoprecipitation
ISCT	International Society for Cellular Therapy
ко	Knock-Out
LAP	Latency-Associated Peptide
LC-MS	Liquid Chromatography/Mass Spectroscopy
LRP5/6	Low-Density Lipoprotein-Receptor-Related Protein 5/6
МАРК	Mitogen-Activated Protein Kinase
MCAM	Melanoma Cell Adhesion Molecule
MgCl <sub>2</sub>	Magnesium Chloride
MMLV	Moloney Murine Leukemia Virus
MSC	Mesenchymal Stem Cell
Msx-2	Msh Homeobox 2
MTA	Mineral Trioxide Aggregate
MTT	Thiazolyl Blue Tetrazolium Blue
MWCO	Molecular Weight Cut-Off
NaCl	Sodium Chloride
Nanog	Nanog Homeobox
NaOH	Sodium Hydroxide
NCP	Non-Collagenous Protein
OC	Osteocalcin
Oct-4	Octamer-Binding Transcription Factor-4

OPG	Osteoprotegerin
OPN	Osteopontin
Osx	Osterix
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PBS <sup>++</sup>	Phoshpate Buffered Saline/1mM Calcium Chloride/1mM Magnesium Chloride
PCR	Polymerase Chain Reaction
PD	Population Doubling
PDGF	Platelet-Derived Growth Factor
PDGFR	Platelet-Derived Growth Factor Receptor
PDL	Periodontal Ligament
PG	Proteoglycan
PIGF-2	Placental Growth Factor-2
PPAR-Y	Peroxisome Proliferator-Activated Receptor-Gamma
PTFE	Polytetrafluoroethylene
q-PCR	Quantitative Real Time-Polymerase Chain Reaction
RANK	Receptor Activator of Nuclear Factor Kappa-B
RANKL	Receptor Activator of Nuclear Factor Kappa-B Ligand
RGD	Argenine-Glycine-Aspartic Acid
rh	Recombinant Human
RLU	Relative Luminescence Unit
RNA	Ribonucleic Acid
RT	Reverse Transcription
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
RunX2	Runt-related Transcription Factor-2 (Alias: CBFA1)
SD	Standard Devation (of the mean)
SDS	Sodium Dodecyl Sulphate
SF	Silk-Fibroin
SHED	Stem Cells from Human Exfoliated, Deciduous Teeth

SIBLING	Small Integrin-Binding Ligand N-linked Glycoprotein
SLRP	Small, Leucine-Rich Proteoglycan
Smad	Sma and Mad Related Family
Sox5	SRY-Box 5
Sox9	SRY-Box 9
T1D	Type 1 Diabetes Mellitus
T2D	Type 2 Diabetes Mellitus
TAZ	Tafazzin
ТВЕ	Tris/Borate/EDTA
TBR	Transforming Growth Factor-β Receptor
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline/0.05% Tween-20
TERT	Telomerase Reverse Transcriptase
TGF-β	Transforming Growth Factor-β
ТМВ	3,3',5,5'-Tetramethylbenzidine
TNF-α	Tumour Necrosis Factor-α
VEGF	Vascular Epidermal Growth Factor
VEGFR	Vascular Epidermal Growth Factor Receptor
Wnt	Wingless Associated Pathways

## **Units of Measurement**

%	Percentage
bp	Base Pairs
°C	Degrees Celcius
cm <sup>2</sup>	Centimetres Squared
Da	Dalton
g	Gravitational Acceleration
g	Gram
h	Hour

kDa	Kilodalton
М	Mole
ΜΩ	Megohm
mg	Milligram
Min	Minute
mL	Millilitre
mM	Millimolar
Ν	Equivalent Concentration
ng	Nanogram
nm	Nanometre
μg	Microgram
μι	Microlitre
μΜ	Micromolar
pg	Picogram
RLU	Relative Luminescence Units
S	Second
V	Volts
v/v	Volume/Volume
w/v	Weight/Volume

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## Chapter 1 - General Introduction

#### **1.1 Background Introduction**

Adult mesenchymal stem cells (MSCs) have been well characterised over recent years and are indispensable constituents in the process of tissue repair and regeneration. Bone is a highly dynamic tissue possessing its own niche of MSCs that enable efficient tissue regeneration in response to tightly regulated biological signals, with growth factors being well characterised in this process. However, these signalling events are disrupted in conditions, such as osteoporosis, diabetes and in implant dentistry, where altered biological signalling induces resorption and thus complicates implant placement.

Currently, the 'gold standard' method for enhancing bone regeneration is to use an autologous bone graft (ABG), most commonly obtained from the posterior ileac crest. However, donor site morbidity and reduced effectiveness in patients with systemic compromising complications, such as diabetes and osteoporosis, has prompted the search for alternatives. ABGs owe their effectiveness to regenerate tissue as they contain physiological concentrations of bioactive growth factors embedded in an extracellular matrix (ECM), to stimulate resident bone marrow-derived, mesenchymal stem cells (BMMSCs) to differentiate down the osteogenic lineage and secrete reparative bone. Bone allografts are alternatives used to alleviate the complications experienced by ABGs, however, these pose a risk of disease transmission and subsequent sterilisation to improve their safety diminishes the grafts osteoinductivity. The use of growth factors clinically to enhance bone repair, notably bone morphogenetic proteins (BMPs), BMP-2 and BMP-7, are regarded as largely ineffective due limited efficacy and cost considerations as a result of the supra-physiological doses required.

Mounting evidence indicates that the ECM of mineralised tissue plays critical roles in supporting the activity of growth factors towards cells. Present knowledge indicates that matrix proteins serve to sequester growth factors and modulate their biological activity. Notably, the matrix proteins biglycan and decorin have been shown to bind avidly to growth factors, such as transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) (Yamaguchi *et al*, 1990), in addition to enhancing the signalling response of BMP-4 (Chen *et al*, 2004) and BMP-2 (Mochida *et al*, 2006), both of which possess roles in differentiation of MSCs to osteoblasts. There are additional reports that signify the importance of extracellular matrix components in orchestrating cell signalling events that enable optimal tissue repair to occur (Berendsen *et al*, 2011).

*In vivo*, dentine is reported to possess bioactive characteristics for augmentation of bone repair. Notably, dentine has been demonstrated to enhance the repair of bone in diabetic rabbits (Gomes *et al*, 2008) and in the tooth sockets of rats (Reis-Filho *et al*, 2012; de Oliveira *et al*, 2013). Recent proteomic studies relating to human dentine have indicated that dentine contains 147 and 289 protein components, respectively (Chun *et al* 2011, Jágr *et al* 2012). Although the presence of numerous growth factors has been confirmed in dentine, a large proportion of the remaining components remain elusive as to their specific purpose within the matrix.

The aims of this Thesis were to assess the bioactive potential for dentine matrix to drive differentiation of BMMSCs towards the osteoblastic lineage. Optimal concentrations of growth factors present within dentine matrix that enhance osteogenic differentiation were assessed and their roles in bioactivity of dentine determined. Additionally, the significance of biglycan and decorin in the bioactivity of dentine was tested. Ultimately, the research undertaken within this Thesis aimed to improve knowledge of the pertinent constituents of dentine ECM to lead to the development of bioactive scaffolds for use in clinical bone repair.

#### 1.2 Stem Cells

#### 1.2.1 Stem Cell Overview

In recent years, the term 'stem cell' has obtained increasing recognition and is discussed widely among literature in relation to to cell therapy and tissue engineering. A stem cell can be described as possessing two properties; the ability to self-renew and to be able to differentiate into a specialised cell with a determined function (Romito and Cobellis, 2015). Stem cells are widely regarded as indispensable entities for the development, maintenance and repair of tissues and organs throughout life (Weissman, 2000; Pekovic and Hutchinson, 2008). The potency of a stem cell can be described as one of four definitions. Totipotent stem cells possess the ability to differentiate into any cell within the body; pluripotent stem cells can differentiate into phenotypes from different germ layers; multipotent stem cells are capable of differentiation into phenotypes from the same germ layer; unipotent stem cells only possess the capacity to differentiate into one type of cell (Larijani *et al*, 2012). Broadly, stem cells are divided into two groups; embryonic stem cells (ESCs) and adult stem cells.

#### **1.2.2** Embryonic Stem Cells (ESCs)

Human ESCs were initially isolated in humans from the inner cell mass of blastocysts (Thomson *et al*, 1998) and possess the ability to indefinitely self-replicate and remain in an undifferentiated state. ECS by definition are termed pluripotent, as they are able to differentiate into cells from all three germ layers *in vitro* and *in vivo* (De Paepe *et al*, 2014). Such sought properties of ESCs demonstrate ideal characteristics for use in tissue engineering and regenerative medicine. However, intense ethical issues relating to ESC isolation and processing negates their use as a suitable candidate for use within these fields and thus alternative options are sought (Behr *et al*, 2010).

Telomeres are sequences of DNA rich in guanine repeats and protect eukaryotic chromosomes against degradation (Blackburn, 2001). During cell replication, telomeres progressively shorten which ultimately results in incomplete chromosomal replication, chromosomal senescence and loss of cell viability (Collins

and Mitchell, 2002). Telomerase reverse transcriptase (TERT), encoded by the *TERT* gene, prevents chromosomal senescence by replenishing telomeres (Blasco, 2005). ESCs express *TERT* and possess high telomerase activity, which decreases following differentiation of ESCs (Saretzki *et al*, 2008). However, telomerase activity is reported to be very low or absent in adult stem cells (Flores *et al*, 2006).

ESCs possess characteristic markers to determine their phenotype. Oct4 is a homeodomain transcription factor (Wu and Schöler, 2014). Oct4 knock-out (KO) in embryos results in loss of pluripotency of the inner cell mass (Nichols *et al*, 1998). In addition, forced expression of Oct4 generates induced pluripotent stem cells from neural stem cells (Kim *et al*, 2009). Nanog is a divergent homeodomian protein additionally associated as a marker of ESCs, as it is expressed in pluripotent cells, however, its expression is absent in differentiated cells (Chambers *et al*, 2003). Nanog expression is also regarded as critical for pluripotency of ESCs as deficiency results in differentiation towards the extra-embryonic endoderm lineage (Mitsui *et al*, 2003). Although ESCs are not the focus of this Thesis, the expression of ESC markers, such as Oct4 and Nanog, have been identified in adult stem cells and are postulated to possess functional roles in determining the differentiation potential (Riekstina *et al*, 2009).

#### 1.2.3 Mesenchymal Stem Cells (MSCs)

The original notion of osteogenic precursor cells was proposed by Friedenstein *et al* in 1968, when cells from the bone marrow of mice could differentiate into bone *in vitro*. A couple of years later, a sub-set of cells from the bone marrow were identified noted to possess clonogeneic behaviour, which were initially referred to as colony forming unit-fibroblastic (CFU-F) (Friedenstein *et al*, 1970) and subsequently were identified in human bone marrow (Castro-Malaspina *et al*, 1980). The term mesenchymal stem cell was coined in 1991, where the CFU-F cells from bone marrow were demonstrated to possess differentiation potential towards cells of bone and cartilage lineage (Caplan, 1991). Since their initial discovery, MSCs have been identified within many tissues of the body including, but not limited to bone marrow, adipose tissue, dental pulp, articular cartilage and liver tissue (Huang *et al*, 2011). MSCs are reported to reside in a specialised microenvironment,

referred to as a niche, which additionally contains ECM and signalling molecules associated with the MSC in question (Spradling *et al*, 2001). However, the precise milieu and composition of the niche varies between different MSCs. The role of the niche is to provide the optimal environment for the balance of quiescent and active cells to be achieved, thus maintaining a constant supply of slowly dividing cells (Chen *et al*, 2011). Upon activation, due to tissue requirement, disease state or trauma, MSCs are able to exit the niche and proliferate and differentiate to produce specialised cells that can contribute to the repair of the compromised tissue.

Generally, it is accepted that stem cells lie predominantly dormant in tissues and that only a select few are active for participation in tissue homeostasis (Kindler, 2005). Stem cells undergo two types of division, as outlined in Figure 1.1. Asymmetric division occurs when a mother cell divides into an identical daughter cell and a daughter "transit" cell, which has lost its indefinite proliferative capability (Potten and Loeffler, 1990). Asymmetric cell division serves as a purpose to allow self-renewal of the mother stem cell in addition to producing a more differentiated cell, therefore contributing to cell maintenance. Symmetric cell division is classed as a mother cell dividing into two duplicate daughter cells, which possess identical characteristics. Such division contributes to self-renewal and subsequent expansion of the stem cell population (Tajbakhsh *et al*, 2009). Stem cell expansion occurs to maintain a sufficiently large pool of undifferentiated cells available for differentiation into lineage specific cells to participate in wound repair (Egger *et al*, 2011).



Figure 1.1 - Schematic image outlining asymmetric division (a) and symmetric division (b) of stem cells. Image adapted from Knoblich (2001).

The process of asymmetric cell division resultantly gives rise to the existence of a highly heterologous population of cells. Heterogeneity of MSCs has been noted in literature reports via observations of clonal populations of MSCs, with three observed phenotypes noted; spindle-shaped, star-shaped and large/flat cells (Muraglia *et al*, 2000; Xiao *et al*, 2010). Extended *in vitro* culture of clonal populations of BMMSCs results in the sequential loss of adipogenic and subsequent chondrogenic differentiation potential, therefore leading to cells with the potency to differentiate towards the osteogenic lineage (Digirolao *et al*, 1999; Muraglia *et al*, 2000). Heterogeneous BMMSCs are thus postulated to consist of highly proliferative, non-committed cells along with cells characterised by low proliferative capability and lineage restriction (Chan *et al*, 2004; Phinney, 2012) (Figure 1.2).



Figure 1.2 - Schematic diagram representing the heterogeneous nature of MSC populations in relation to lineage commitment hierarchy. Clonal progenitors sequentially lose tri-potent differentiation potential through bi-potency down to uni-potent cells. Adapted from Phinney (2012).

As a means of MSC standardisation, the International Society for Cellular Therapy (ISCT) recommended the minimal criteria for human MSC identification. Firstly, MSCs are plastic adherent in standard culture conditions. Secondly, fluorescenceactivated cell sorting (FACS) analysis indicates the presence of cell surface markers CD105, CD73 and CD90. The absence of CD34 (hematopoietic progenitor), CD45 (pan-leukocyte), CD14 and CD11b (monocyte and macrophage), CD79a and CD19 (B-cell); and HLA-DR (post MSC stimulation) markers, are also definitive of MSCs. Finally, in addition to the other criteria, MSCs must also be able to undergo *in vitro* differentiation into osteoblasts, adipocytes and chondrocytes, determined by Alizarin red/von Kossa, Oil Red O and Alcian blue/collagen type II immunohistochemical staining, respectively (Dominici *et al*, 2006).

Although the current definition of an MSC is to fulfil all three requirements outlined by Dominici *et al* (2006), the expression of cell surface markers is often solely used to characterise MSC populations. However, evidence suggests that the expressions of markers associated with MSCs are not faithfully associated with this cell type. The markers, CD90 and CD105, have been shown to demonstrate positive expression in skin-derived fibroblasts (Alt *et al*, 2011). Furthermore, endothelial cells obtained from the umbilical vein are reported to co-express CD105 and CD73 (Narravula *et al*, 2000). The notion that MSCs should be CD34 negative has also provoked controversy, as CD34 expressing cells from the bone marrow have been demonstrated to differentiate into chondrocytes, adipocytes and osteoblasts (Kaiser *et al*, 2007). As a result of the reported discrepancies in the markers used to identify MSCs, additional markers have been investigated to aid in the determination of MSC populations.

CD146 (Melanoma Cell Adhesion Molecule; MCAM) has been identified in MSCs derived from bone marrow (Shi and Gronthos, 2003) and its expression in clonallyderived BMMSCs, is around 2-fold higher in multi-potent clones, compared to unipotent clones (Russell *et al*, 2010). As mentioned previously, ECS markers are also implicated to be expressed in MSC populations, notably Oct4 and Nanog. Although Oct4 and Nanog expression is negative in osteoblasts, their expression is present in MSCs prior to differentiation (Arpornmakelong *et al*, 2009).

Despite the efforts to determine markers that strictly identify MSCs, no definitive marker has of yet been identified (Feng-Juan *et al*, 2014). Therefore, identifying MSCs *in vitro* can be challenging, especially when considering the reported heterogeneity in differentiation potential (Chan *et al*, 2004; Phinney, 2012). A method to isolate a more homologous population of MSCs from an otherwise heterogeneous population is via adhesion to fibronectin, through interactions with  $\alpha$ 5 $\beta$ 1 integrins on the cell surface (Wu *et al*, 1993). MSCs expressing  $\alpha$ 5 $\beta$ 1 integrins on their surface are correlated with greater proliferative capacity and enhanced colony forming efficiency (Jones and Watt, 1993; Jones *et al*, 1995). Consequently, primitive cells have been preferentially selected from heterogeneous populations via adhesion to fibronectin (Jones *et al*, 1995; Dowthwaite *et al*, 2004; Waddington *et al*, 2009).

#### 1.3 Regulation of Osteogenic Differentiation

#### 1.3.1 Osteoblastic Differentiation of MSCs

The differentiation of MSCs into osteoblasts, the cells responsible for the synthesis of bone, is a complex process involving the interplay of various signalling molecules, transcription factors and transcribed proteins. The transcription factor RunX2 (runt-related transcription factor-2, also known as core-binding factor subunit  $\alpha$ , Cbfa1), is widely regarded as the master gene required for osteogenic differentiation. In mice, RunX2 deficiency has been shown to result in the inability to form osteoblasts (Otto *et al*, 1997). In humans, a non-sense mutation of the RunX2 gene that is transcribed to a truncated RunX2 protein results in the autosomal dominant bone disorder cleidocranial dysplasia (Zhang *et al*, 2000). Activated RunX2 binds to promoters of osteoblast-associated genes, including osteopontin (OPN), type I collagen, bone sialoprotein (BSP) and osteocalcin (OC) (Ducy *et al*, 1997; Kern *et al*, 2001).

There are numerous literature reports demonstrating that RunX2 mRNA expression is up-regulated during differentiation of MSCs (Tsuji et al, 1998; Banjeree et al, 2001; Gu et al, 2012), however, there is contradictory evidence relating to RunX2 expression and differentiation of human MSCs to osteoblasts. It has been noted that human MSCs constitutively express RunX2 mRNA, although a correlation between RunX2 expression and the generation of an osteoblast phenotype do not demonstrate a positive correlation (Ven den Bos, 1998). In addition, induction of osteoblast differentiation in human MSCs by dexamethasone is reported to increase OC expression with no changes in RunX2 expression observed (Shui et al, 2003). Phosphorylation of RunX2 protein and increased DNA binding is postulated to induce osteogenic differentiation without altering RunX2 mRNA expression levels (Prince et al, 2001; Shui et al, 2003). On the contrary, two isoforms of RunX2 mRNA are reported to exist; type I demonstrates constitutive expression in non-osseous mesenchymal tissues and osteoblast progenitors, however, type II RunX2 is reported to increase expression during osteoblast differentiation (Banerjee et al, 2001; Sudhakar et al, 2001). Consequently, the activation of RunX2 is proposed to

undergo regulation on several levels, including transcription, translation and protein activation.

The expression and activation of RunX2 is regulated by additional transcription factors. Msx2 is a homeobox protein, which prevents osteogenic differentiation and switches cells to a state of proliferation (Dodig *et al*, 1999). Msx2 represses the OC promoter in rats (Newberry *et al*, 1997) and is postulated to prevent osteoblast differentiation by repressing the transcription of RunX2 (Shirakabe *et al*, 2001). Dlx5 is another homeobox protein which regulates RunX2 and acts antagonistically to Msx2. Dlx5 overexpression stimulates the promoter region of type II RunX2 isoform, whereas Msx2 acts to suppress the promoter (Lee *et al*, 2005). Dlx5 alleviates the suppressive action of Msx2 and thus promotes osteoblast differentiation (Shirakabe *et al*, 2001).

The transcription factor, Taz, induces increased activation of the OC promoter via RunX2, whereas this increase is not observed in a dominant negative phenotype of Taz (Cui *et al*, 2003). Taz co-activates the expression of RunX2 dependent genes whilst simultaneously inhibiting adipogenesis by suppressing PPAR-Y related gene expression, thus suggesting Taz acts determine the fate of multipotent MSCs (Hong *et al*, 2005).

Osterix (Osx) is a transcription factor that acts downstream of RunX2, as Osx-null mice do not form bone despite expression of RunX2, in addition to lack of OSx expression in RunX2 null mice. Osx null pre-osteoblasts also express chondrocyte marker genes, Sox9, Sox5 and collagen type II (Nakashima *et al*, 2002). Osx expression is also inducible by BMP-2 in RunX2 deficient MSCs (Matsubara *et al*, 2008). Expression of Osx stimulates osteoblast maturation by increasing expression of mature osteoblast markers, including OC, BSP and type I collagen (Komori, 2006; Choi *et al*, 2011).

Figure 1.3 summarises the key transcription factors associated with osteoblast differentiation from MSCs.

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Figure 1.3 - Schematic diagram of the main transcription factors that govern osteogenic differentiation of MSCs. TAZ inhibits adipogenic differentiation potential of MSCs and commits cells to the osteogenic lineage. Activation of RunX2 is required for differentiation of committed osteoprogenitors to pre-osteoblasts and subsequently activates Osx, which induces maturation to osteoblasts. Adapted from Hughes *et al*, (2006).

#### **1.3.2** Signalling Factors Associated with Osteogenic Differentiation

#### **1.3.2.1** Transforming growth factor- $\beta$ (TGF- $\beta$ )

Transforming growth factor- $\beta$  (TGF- $\beta$ ) proteins belong to a super gene family comprised of five closely related molecules, in addition to homologous cousins, which include BMPs (Allori *et al*, 2008). Three isoforms of TGF- $\beta$  (TGF- $\beta_1$  – TGF- $\beta_3$ ) are present in mammals and are highly homologous, exerting their effects through type I and type II serine-threonine kinase receptors (TBRI and TBRII, respectively). TGF- $\beta$  binds to a tetrameric receptor comples consisting of two of each TBRI and TBRII receptors, after which TBRII phosphorylates TBRI. In Smad-dependent TGF- $\beta$ signalling, activated TBRs induce phosphorylation of Smad proteins 2 and 3. Subsequently, a complex with Smad4 is formed which translocates to the cell nucleus to regulate target gene expression (Nakao *et al*, 1997; Chen *et al*, 2012; Huang *et al*, 2012a).

All three isoforms of TGF- $\beta$  are expressed at sites of intramembranous and endochondral ossification (Horner *et al*, 1998). Sub-periosteal injections of TGF- $\beta_1$ and TGF- $\beta_2$  motivate endochondral ossification, along with TGF- $\beta_2$  stimulating expression of TGF- $\beta_1$  (Joyce *et al*, 1990). TGF- $\beta_1$  is a potent stimulator of proliferation (Zhang *et al*, 2015a) and migration (Tang *et al*, 2009) of BMMSCs *in vitro*. TGF- $\beta_1$  does not induce osteoblastic differentiation of BMMSCs (Katagiri *et al*, 1994); on the contrary, TGF- $\beta_1$  is noted to repress expression of RunX2 (Kang *et al*, 2005), in addition to exerting inhibitory activities against osteogenesis at later stages of differentiation (Maeda *et al*, 2004). It is, however, postulated that TGF- $\beta_1$ induces proliferation BMMSCs and osteoblastic precursors to increase the numbers of available cells available to participate in osteogenic differentiation (Centrella *et al*, 1994; Rodrigues *et al*, 2010).

#### **1.3.2.2** Bone Morphogenetic Proteins (BMPs)

BMPs were initially identified as components of organic bone matrix; and were able to induce formation of ectopic bone, by Urist (1965). Since their initial discovery, over twenty members have been identified and represent the largest sub-group of the TGF- $\beta$  superfamily (Kawabata *et al*, 1998; Wang *et al*, 2014). Constituting such a

wide array of signalling molecules, BMPs exert various processes on cells including proliferation (Stewart et al, 2010), apoptosis (Zou et al, 1996; Lagna et al, 2006); and differentiation (Kobayashi et al, 2005; Luther et al, 2011). BMPs bind to BMP receptor II (BMPR-II) on the cell surface, which in turn reacts with BMP receptor I (BMPR-I). Activated BMP receptors in turn phosphorylate and activate Smad proteins 1, 5 and 8, which form complexes with Smad4 and translocate to the nucleus and interact with the transcription factors RunX2, Osx Dlx5 and Msx-2 to regulate osteogenic differentiation (Canalis, 2009; Lin and Hankerson, 2011; Rahman et al, 2015). BMPs are also able to induce osteogenesis independently of Smads, via activation of the mitogen-activated protein kinase (MAPK) signalling cascade, where induction of the MAPK pathway results in phosphorylation and activation of RunX2 (Greenblatt et al, 2013). Of the BMPs, BMP-2, BMP-4 and BMP-7 are reported to have potent activity in influencing osteogenic differentiation of rodent cells (Hughes et al, 1995; Franceschi et al, 2000; Hassan et al, 2006), in addition to promoting osteogenesis of human MSCs (Gori et al, 1999; Lavery et al, 2008; Shen et al, 2010).

#### 1.3.2.3 Wnt

Wnt (Wingless) is a signalling cascade that consists of around twenty cysteine-rich glycoproteins, which can act in a canonical or non-canonical manner. In canonical signalling, Wnt ligands bind to transmembrane receptor Frizzled (Fzd) and correceptor low-density lipoprotein-receptor-related protein 5/6 (LRP5/6). Binding of Wnt ligands to these receptors causes intracellular accumulation of  $\beta$ -catenin, which can enter the nucleus and interact with the transcription factor T-cell factor/lymphoid enhancer factor, permitting the activation of transcription of target genes (Lin and Hankerson, 2011; Wanger *et al*, 2011).  $\beta$ -catenin protein levels are acknowledged to be up-regulated in osteoblast precursors, whereas  $\beta$ -catenin inactivation results in the chondrocytic differentiation of cells (Day *et al*, 2005). Loss of function mutations in LRP5 causes osteoporosis-pseudoglioma syndrome (Levasseur *et al*, 2005). Conversely, gain of function mutations in LRP5 result in increased bone density, accompanied by elevation of markers of bone formation, including OC (Boyden *et al*, 2002). Recombinant BMP-2 induces up-

regulation of the Wnt ligands, Wnt-7a, Wnt-10b, Wnt-11 and Wnt-13, in addition to LRP-6 receptor. Inactivation of the  $\beta$ -catenin gene results in significantly reduced chondrogenic differentiation of mesenchymal cells, following BMP-2 administration (Chen *et al*, 2007). In addition, expression of canonical Wnt proteins has been shown to result in up to a 5-fold increase of RunX2 promoter activity (Gaur *et al*, 2005).

#### **1.3.2.4** Fibroblast Growth Factors (FGFs)

Fibroblast growth factors (FGFs) refer to a family of at least twenty-three highly conserved, monomeric peptides (Wiedłocha and Sørensen, 2004), which act through four related tyrosine kinase receptors (FGFR1-FGFR4) (Huang et al, 2007). Studies show that inactivated FGFR2 causes skeletal dwarfism accompanied with reduced bone mineral density (BMD) compared to controls, accompanied with decreased osteoblast proliferation (Yu et al, 2003). Haploinsuffiency of FGF-2 leads to decreased BMD compared to wild type, which is rescued with the application of exogenous FGF2. Reduced mRNA secretion of RunX2 also correlates with FGF2 +/and FGF -/- (Naganawa et al, 2006), suggesting crosstalk between RunX2 and FGF signalling. This relationship was supported by Naganawa et al (2008), who demonstrated that FGF2 -/- osteoblasts experienced reduced BMP-2 induced nuclear accumulation of phosphorylated Smad 1/5/8, along with diminished colocalisation to RunX2. Furthermore, FGF2-/- BMMSCs experience reduced  $\beta$ -catenin accumulation, which is rescued following application of exogenous FGF-2 (Fei et al, 2011). FGFR3 KO also causes a reduction in bone mineralisation and lack of trabecular connectivity (Valverde-Franco et al, 2004).

#### **1.3.2.5** Vascular Epidermal Growth Factor (VEGF)

Vascular epidermal growth factor (VEGF) is a family of seven closely related growth factors that exert biological signalling effects through three tyrosine kinase receptors (VEGFR1 – VEGRF3) (Roy *et al*, 2006). VEGF is critical for embryological development, with heterozygous loss of a VEGF allele in ESCs resulting in impaired angiogenesis and severe developmental abnormalities (Ferrara *et al*, 1996). VEGF plays critical roles in the development of bone as VEGF suppression almost completely subdues blood vessel penetration into the epiphyseal growth plate,

affiliated with hindered trabecular bone formation and enlargement of the hypertrophic chondrocyte zone (Gerber *et al*, 1999). Exogenous VEGF administration enhances angiogenesis and callus maturation, whereas neutralising VEGF decreases angiogenesis and callus mineralisation (Street *et al*, 2002). VEGF is regulated by the hypoxic inducible factor 1 (HIF-1) pathway, where an oxygen-related subunit, HIF-1 $\alpha$ , accumulates and dimerises under low oxygen tension, mediating changes in hypoxia-inducible genes (Dai *et al*, 2007). Overexpression HIF-1 $\alpha$  in osteoblasts results in raised levels of VEGF, accompanied with the development of highly vascularised and dense bones (Wang *et al*, 2007). VEGF mRNA transcription is up-regulated in fibroblasts overexpressing RunX2 (Zelzer *et al*, 2001), contributing to the importance of angiogenesis in endochondral bone formation.

#### 1.3.2.6 Insulin-like Growth Factor (IGFs)

Insulin-like growth factors (IGFs) are a family of two polypeptides (IGF-I and IGF-II) that act through two tyrosine kinase IGF receptors (IGFR-I and IGFR-II), which elicit cellular proliferation and growth. Osteoblasts express both IGF-I and IGF-II, although the former is noted to be more potent (Allori *et al*, 2008; Arvidson *et al*, 2011). IGF has been shown to influence bone formation by acting on differentiated cells, where application of IGF to mature osteoblasts increases collagen type I expression. However, IGF does not alter gene expression of RunX2, alkaline phosphatase (ALP), type I collagen or OC in human BMMSCs (Thomas *et al*, 1999). IGF-1 has also been demonstrated to enhance phosphorylation and stabilisation of  $\beta$ -catenin (Playford *et al*, 2000).

#### **1.3.2.7** Platelet-Derived Growth Factor (PDGFs)

Platelet-derived growth factors (PDGFs) are a family of growth factors consisting of 3 isoforms; homodimers composed of polypeptide chains PDGF-A (PDGF-AA) and PDGF-B (PDGF-BB); and a heterodimer (PDGF-AB) (Heldin and Westermark, 1999). PDGF signals primarily through PDGF receptor  $\alpha$  (PDGFR $\alpha$ ), whereas PDGF-BB predominantly signals via PDGF receptor  $\beta$  (PDGFR $\beta$ ) and signals mainly through the MAPK pathway (Donovan *et al*, 2013). Deletion of PDGFR $\beta$  in MSCs induced to differentiate to osteoblasts results in enhanced RunX2 and OC expression (Tokunaga *et al*, 2008). PDGF-BB receptor inhibition in human MSCs is reported to decrease proliferation, however, possess no influence on osteogenic differentiation (Kumar *et al*, 2010). In addition to enhancing proliferation of MSCs, PDGF acts as a chemotactic agent for human MSCs (Fiedler *et al*, 2002).

# 1.4 Extracellular Matrix (ECM) Components Associated with Mineralised Tissues

### 1.4.1 Small, Leucine-Rich Proteoglycans (SLRPs)

Small leucine-rich proteoglycans (SLRPs) were originally identified as proteoglycans consisting of protein cores of around 36-45 kDa. As their names suggest, the protein cores contain tandem leucine-rich repeats and undergoes translational modification to possess covalently bound glycosaminoglycan (GAG) chains of varying composition and length (lozzo and Murdoch, 1996; lozzo and Schaeffer, 2010; Nikitovic *et al*, 2012). The SLRP family currently encompasses eighteen genes which are further classified into five sub-families, based on chromosomal organisation, N-terminal cysteine-rich clusters and functional similarities (McEwan *et al*, 2006; Schaeffer and lozzo, 2008; lozzo *et al*, 2011).

Following synthesis, SLRPs are secreted into the pericellular matrix where they can bind to ECM constituents or remain free within the matrix, although the association of SLRPs with other matrix constituents is regarded as a strictly pre-determined process (Henry *et al*, 2001; Gruber *et al*, 2002; Vanderploeg *et al*, 2012). Members of the SLRP family have been identified as being able to promiscuously interact with a plethora of factors within the ECM, including TGF- $\beta_1$  (Hildebrand *et al*, 1994), BMP-4 (Chen *et al*, 2004), PDGF (Nili *et al*, 2003) and IGF-1 (Schönherr *et al*, 2005). Additionally, some SLRPs are implicated as being able to bind to matrix collagens (Scott, 1996; Reed and Jozzo, 2002; Zhang *et al*, 2006).

## 1.4.2 Biglycan and Decorin

Biglycan and decorin are members of the class I sub-family of SLRPs. Biglycan consists of a 45 kDa protein core containing tissue-specific GAG chains composed of either dermatan sulphate (DS) or chondroitin sulphate (CS), which are covalently attached at the N-terminus of the core protein (Choi *et al*, 1989; Roughley and

White, 1989; Bianco et al, 1990). Non-glycosylated forms of biglycan have been discovered, however, in articular cartilage (Roughley *et al*, 1993) and intervertebral discs (Johnstone et al, 1993). Structurally similar to biglycan, decorin consists of a core protein of around 42 kDa containing a single GAG chain of either DS or CS, covalently linked to a serine residue near the N-terminus of the protein (Krusius and Ruoslahti, 1986; Mann et al, 1990). Schematic structures of biglycan and decorin are outlined in Figure 1.4. Human biglycan and decorin are encoded on chromosomes X and twelve, respectively (McBride et al, 1990). Initial determination for the roles of biglycan in bone development were speculated when patients suffering from Turner's syndrome (females devoid of a second X chromosome), were observed to be shorter in stature and possess low levels of biglycan, contrary to patients possessing supernumerary X chromosomes that presented with increased limb length and higher biglycan levels. Subsequent in vivo investigations into the targeted deletion of the biglycan gene in mice resulted in delayed and decreased bone growth compared to wild-type animals, accompanied with the inability to achieve peak bone mass attributable to diminished osteoblast numbers (Xu et al, 1998). In vivo KO of decorin in mouse models does not incur the osteopenic phenotype as displayed in biglycan KO models, however, dual KO of both biglycan and decorin results in a marked osteopenic phenotype (Corsi et al, 2002). BMMSCs derived from biglycan and decorin dual KO mice are additionally noted to result in over-activation of the TGF- $\beta$  signalling pathway due to unregulated sequestration. Consequently, osteoprogenitors undergo apoptosis and result in decreased bone formation (Bi et al, 2005). In vitro, the depletion of decorin and biglycan from dentine via immunoprecipitation results in a 47-fold reduction in binding affinity of TGF- $\beta_1$ , thus indicating a role for sequestration and protection of biological activity within dentine (Baker *et al*, 2009).



Figure 1.4 - Schematic diagram of the structure of the SLRPs biglycan and decorin. Core proteins contain leucine-rich repeats flanked either side by disulphide bonds. DS and/or CS GAG chains are covalently linked to the core protein at the amino terminus end. Image adapted from Embery *et al*, (2001).

#### 1.4.3 Roles for Biglycan and Decorin in Mineralised Tissues

The importance of SLRPs in mineralisation can be determined as a result of their presence within mineralised tissues. Within mineralised matrices, biglycan and decorin are predominantly substituted with CS GAGs, however, in soft connective tissues they are usually associated with DS GAGs (Fisher *et al*, 1989; Bratt *et al*, 1992).

Regarding mineralisation, CS GAGs are reported to possess around a 5-fold greater binding capacity for calcium than DS GAGs (Embery *et al*, 1998). In bone, both biglycan and decorin are also identified with decorin being localised in the pericellular matrix, whereas biglycan demonstrates a more homogeneous distribution throughout the matrix (Ingram *et al*,1993). During osteogenic differentiation of alveolar bone-derived cells, biglycan is expressed during cell proliferation and again at the onset of mineralisation, whereas decorin expression remains consistent. DS represent up to around 70% of total sulphated GAGs during proliferation, whereas CS constitutes 100% of GAGs during mineralisation (Waddington *et al*, 2003a). This observation correlates largely with that seen in dentine, where biglycan and decorin substituted with GAGs are present. However, a shift in profile from DS-substituted SLRPs in pre-dentine to predominantly CS-substituted SLRPs in the pre-dentine/dentine interface to completely CS-substituted SLRPs in calcified dentine is noted, therefore suggesting differential functions in directing mineralisation (Waddington *et al*, 2003b).

#### 1.4.4 Biglycan and Decorin in the Modulation of Cell Signalling Events

Mounting evidence is also for ECM components modulating cell signalling events. Biglycan has been affiliated with enhancing osteogenic responses to BMP-4, with biglycan deficiency demonstrating attenuated RunX2 expression, which was restored following reintroduction of biglycan (Chen *et al*, 2004). Treatment of murine C2C12 myoblasts with BMP-2 induces lineage conversion into osteoblasts, accompanied with increased synthesis of decorin. Moreover, BMP-2 signalling was diminished in decorin null myoblasts, suggesting the existence of a feedback mechanism (Gutierrez *et al*, 2006). More recently, enhancement of the canonical Wnt signalling pathway via biglycan has been established, by interacting with Wnt ligand Wnt3a in addition to LRP6 via its core protein. Furthermore, biglycan deficiency results in lessened Wnt3a retention and blunts Wnt-induced LRP6 phosphorylation (Berendsen *et al*, 2011). Consequently, both biglycan and decorin are postulated to possess modulatory activity in cell signalling events.

#### 1.5 Bone Repair

#### **1.5.1** Fracture Healing of Bone

Bone tissue has a high regenerative potential *in vivo* and although not fully elucidated, the process of endogenous bone repair has been characterised as a

complex process consisting of a tightly regulated interplay between various signalling mechanisms and cell types.

When a bone fracture occurs, bone architecture in addition to the local blood vessels become damaged, leading to activation of the clotting cascade and the development of a haematoma to aid in stabilisation of the damaged tissue (Kolar et al, 2010). The haematoma is considered to play a critical role in the bone repair process, as haematoma transplanted into the sub-periosteum, in addition to in muscle, produces new bone tissue after 4 days (Mizuno et al, 1990). The arrival of neutrophils at a fracture site occurs around 24 h after injury, where their primary function is to prevent infection by removing debris from the wound (Simpson and Ross, 1972). However, they also function as a secretor of mediators, including interleukin-6 (IL-6) and chemokine (C-C motif) ligand-2 (CCL-2) to recruit monocytes and macrophages (Hurst et al, 2001; Xing et al, 2010). Recruited macrophages further secrete a plethora of chemotactic mediators including tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6 and CCL2 to recruit locally residing MSCs and osteoprogenitor cells to the site of injury (Einhorn et al, 1995; Kon et al, 2001; Bielby et al, 2007). The presence of platelets within the fracture haematoma provides an abundant source of growth factors, such as PDGF, TGF- $\beta_1$ , VEGF and IGF-1 (Eppley *et al*, 2004; Van Dan Dolder *et al*, 2006).

Penetration of newly formed blood vessels into the haematoma permits the infiltration of MSCs and osteoprogenitor cells that initiate the reparative phase (Dimitriou *et al*, 2005; Marsell *et al*, 2009). Repair of fractured bone tissue occurs predominantly via indirect healing, by mechanisms known as endochondral ossification and intramembranous ossification (Gerstenfeld *et al*, 2006). Endochondral ossification mainly occurs adjacently to the fracture site periosteum, where mechanical stability is low and is driven by low oxygen tension (Dimitriou *et al*, 2005). Initially, highly proliferative mesenchymal-derived cells are present within the fracture callus as soon as 3 days post-fracture; and undergo chondrogenesis from around 7-21 days post-fracture to produce a soft callus (Iwaki *et al*, 1997), characterised by increased expression of Sox9 and type II collagen (Einhorn, 1998). Chondrocytes proliferate rapidly for around 10-14 days, after which they undergo

apoptosis (Lee *et al*, 1998; Gibson, 1998). The cartilaginous matrix enhances mechanical stability of the fracture repair site (Claes *et al*, 1997), which promotes vascularisation of the callus (Claes *et al*, 2002), subsequent re-establishment of oxygen tension, pH and nutrient supply permitted by the budding of endothelial cells from nearby capillaries to generate new blood vessels, induced by VEGF, PDGF and FGF (Ai-Aql *et al*, 2008). Vascularisation provides a route of entry for MSCs which differentiate into osteogenic cells and replace the soft callus with woven bone, which subsequently remodels to become regenerated bone (Dimitriou *et al*, 2005; Marsell *et al*, 2009).

Concomitantly, the process of intramembranous ossification occurs at the fracture periphery, where committed osteoprogenitor and undifferentiated MSCs from the periosteum form bone directly without a cartilaginous intermediate (Einhorn, 1998; Carano and Filvaroff, 2003). Intramembranous ossification is dependent on the upregulation of expression of RunX2 and Osx in MSCs to differentiate into osteoblasts (Takarada *et al*, 2016); and is driven by the presence of various growth factors, such as TGF- $\beta_1$ , BMP-2, BMP-4 and BMP-7 (Geiser *et al*, 2005; Bandyopadhyay *et al*, 2006).

#### 1.5.2 Clinical Issues Associated with Bone Repair

#### 1.5.2.1 Alveolar Ridge Augmentation

Tooth extraction is a very common procedure in dentistry with healing of the extraction socket being by large without incident. However, the extraction of one or more teeth from the alveolar ridge typically results in loss of mass of the alveolar ridge, in addition to structural and compositional changes to the soft tissues that superimpose the extraction socket. Removal of single premolars and molars in adult patients results in around a 50% reduction in alveolar ridge width with two thirds of this reduction occurring 3 months post-extraction (Schropp *et al*, 2003; Botticelli *et al*, 2004). It is common for an extracted tooth to be replaced with an artificial implant, which is placed into the resulting socket in the bone. However, the resorption of the alveolar ridge presents a challenge with regards to adequate bone
mass being present to permit the placement of an implant with the potential for long-term survival.

#### 1.5.2.2 Non-Union Fractures

Bone fractures have a generally predictable outcome, however, there are conditions in which the normal healing process is hindered in some way. Fractures can sometimes take longer to heal than anticipated (termed delayed union) or do not heal at all, due to the termination of normal biological healing processes whereby a union does not occur (non-union) (Griffin *et al*, 2011). It has been suggested that a lower number of osteocyte progenitor cells is present at non-union sites in the tibia, compared with non-fractured sites (Hernigou and Beaujean, 1997). It is also claimed that this low abundance of cells is a result of a decreased vascularity that is present at non-union fracture sites (Brighton and Hunt, 1991). Although there are discrepancies in reports which comment upon the incidence of non-union fractures, they are estimated to account for around 2.5% of long bone fractures (Phieffer and Goulet, 2006), although this figure rises dramatically up to 46% when vascular injury is associated with the fracture (Dickson *et al*, 1994). Non-union fractures present numerous complications for patients, most notably a loss of independence and reduced productivity (Aaron *et al*, 2004).

#### 1.5.2.3 Osteosarcoma

Osteosarcoma represents 0.2% of newly diagnosed cancer, most diagnoses being made from tumours in the long bone of the arms and legs. Amputation was considered the standard method of treatment however now 90% of osteosarcoma cases are treated with excision of the affected tissue and thus preservation of the limb as a whole (Chen *et al*, 2005). Bone resection in the craniofacial region poses further implications, as psychological scarring occurs in addition to loss of function of the tissue (Bhumiratana and Vunjak-Novakovic, 2011). Although mechanical loading stimulates bone growth, the stresses and strains present in craniofacial bones are not yet fully understood and thus encouragement of bone growth poses a further challenge (Herring and Ochareon, 2005).

#### 1.5.2.4 Osteoporosis

Osteoporosis is a highly prevalent disease reported to affect around 75 million people worldwide (Durnell et al, 2011); and is characterised as an imbalance between bone remodelling and bone resorption, resulting in a reduction in BMD and disrupted bone architecture (Sandhu and Hampson et al, 2011). Osteoporosis is defined as a BMD of greater than 2.5 standard deviations below the young adult mean in a female population aged between 20 and 40 years of age (Lane, 2006). In females, ovarian senescence occurs gradually between the fourth and fifth decades of life leading to menopausal onset and a marked reduction in oestrogen production (Al-Azzawi and Palacios, 2009). Evidence indicates oestrogen influences several signalling pathways that regulate physiological turnover of bone. Receptor activator of nuclear factor Kappa-B (RANK) is a membrane-bound receptor present on the surface of osteoclast precursors, stimulated by RANK ligand (RANKL) (Michael et al, 2005). Binding of RANKL to RANK results in the phosphorylation and subsequent inactivation of Bad and caspase-9, thus preventing apoptosis (Wong et al, 1999). Oestrogen has been demonstrated to suppress the release of RANKL from osteoblasts (Eghbali-Fatourechi et al, 2003), thus promoting osteoclast apoptosis and aiding the maintenance of normal bone turnover. Recently, oestrogen has also been demonstrated to influence osteogenic signalling; estradiol administration to osteogenic precursor cells from mice enhanced BMP-4 induced expression of RunX2, Osx and OC expression, with BMP-4 induced mineralisation being facilitated by oestrogen treatment (Matsumoto et al, 2013).

## 1.5.2.5 Diabetes Mellitus

Diabetes is a chronic condition, which is estimated to affect around 300 million people globally (Sherwin and Jastreboff, 2012). A diabetic disease state has been implicated in the hindrance of bone turnover and repair. Diabetic patients are reported to be at an increased bone fracture risk compared with the general population, most notably at the hip (Khazai *et al*, 2009). Diabetes also causes an increase in the healing duration, with displaced fractures being reported to take 187% of the normal expected fracture healing time of non-diabetic patients (Loder, 1988). Many reports have commented upon the potential mechanisms for hindered

bone repair in diabetics. Advanced glycation end-products (AGEs) accumulate in a variety of tissues as a natural process in ageing, however hypergycemia that occurs in diabetic patients results in hastened production of AGEs in tissues (Yamaguchi and Sugimoto, 2011). Previous reports have indicated that AGEs influence cellular behavior, with it being demonstrated that AGEs diminish the proliferative capability of MSCs, encourage apoptosis and prevent differentiation into osteoblasts, in addition to chondrocytes and adipocytes (Kume *et al*, 2005). Type I diabetic (T1D) mouse models have demonstrated that hyperglycemic mice undergoing tibia fracture followed by intramedullary pin fixation present a significantly higher number of osteoclasts, compared to mice with normal glycemic levels. Furthermore, mRNA expression levels of RANKL are noted to be raised, preventing osteoclast apoptosis (Kayal *et al*, 2007), supporting the notion that there is a relationship between T1D and osteopenia in up to 55% of cases (Chen *et al*, 2013).

The pathology of type II diabetes (T2D) is more complex. Patients with T2D are noted to have higher BMDs than those suffering with T1D, although the fracture risk in the hip is elevated by 1.4 fold in T2D patients (Vestergaard, 2007). High glucose in conjunction with TNF- $\alpha$  induces apoptosis of MG-63 human osteoblastlike cell lines and thus is postulated to result in delayed fracture healing (Sun *et al*, 2016).

#### **1.5.3** Interventions for Enhancing Bone Repair

## 1.5.3.1 Autologous Bone Grafts (ABGs)

As a result of the various complications associated with hindered bone healing, several technologies are in clinical practice to reduce the burden of bone defects, each with their own advantages and disadvantages.

Bone grafting is a procedure involving the placement of whole bone or bone substitute material within the bone defect to stimulate incorporation of the graft or the generation of bone tissue. ABGs, where bone is harvested from and is placed in the same patient, are the most common bone grafting procedure (Panagiotis, 2005). ABGs are considered the "gold standard" treatment for bone defects, as they possess the required properties necessary for complete bone healing; osteoinduction (stimulation of osteoprogenitor cells to differentiate into osteoblasts), osteogenesis (deposition of bone material by osteocytes) and osteoconduction (presence of a scaffold for new bone development). ABGs are able to express these properties as they contain growth factors, osteoprogenitors, and a scaffold, respectively. ABGs are also advantageous in the respect that as they are obtained from the patient with whom they are to be implanted, they are also histocompatible and non-immunogenic, therefore the risk of infection transmission and immune-rejection are minimised (Dimitriou *et al*, 2011).

The most common site for ABGs to be harvested from is the ileac crest, with reports indicating that volumes of yieldable bone ranging between 5-72cm<sup>3</sup> for the anterior and 25-88cm<sup>3</sup> for the posterior ileac crest (Ahlmann *et al*, 2002). However, the excellent properties attained by ABGs from the ileac crest along with large obtainable volumes are overshadowed by operative complications of the procedure. In the case of patients undergoing an ABG for large bone defects prior to the placement of a dental implant, an additional surgical procedure is involved to obtain the grafting material. Moreover, the use of general anaesthesia, intraoperative complications and post-operative pain negate the use of ABGs for treating bone defects (Brugnami *et al*, 2009). Success rates of ABGs for implant placement are generally high, with reports indicating that using a graft overlay technique of bone grafting typically result in 84-97% success rates at 6 months or more post-operation (Clementini *et al*, 2011). ABGs have also demonstrated successful treatment of non-union fractures, with tibia unions being reported to be between 87-100% following ABG treatment (Sen and Miclau, 2007).

Despite the overall good rates of success for autologous bone grafts, there are contra-indications that result in attenuated rates of success. Diabetes is recognised as a significant factor in the chance of graft survival (Kaing *et al*, 2011), with one study revealing a failure rate of 75% for bone grafts in diabetic patients (Schwartz-Arad *et al*, 2005). In addition, osteoporosis is indicated as a risk factor for alveolar bone augmentation via grafting. Implant survival rate for post-menopausal female patients has been reported to be 43.7%, compared to 80% for male patients (Kramer *et al*, 1999).

#### 1.5.3.2 Bone Allografts

Allografts of bone are obtained from the same species and offer the benefits of ABGs including osteoconduction and osteoinduction, but without the associated issues of donor site morbidity and pain associated with their harvest. After implantation, the physiological process of bone remodelling occurs and eventually the graft is resorbed and replaced with bone tissue from host origin. However, issues relating to disease transmission and immune-rejection are raised with allograft formulations. Transmission of hepatitis C and human immunodeficiency virus (HIV) have been well documented in allograft transplants (Delloye *et al*, 2007). As a result of the risks associated with disease transmission, allografts are required to undergo extensive sterilisation, typically by irradiation. However, these procedures are reported to diminish the mechanical integrity (Cornu et al, 2000) and osteoinductive properties (Han et al, 2008). As a result, they are associated with increased unpredictability compared to autografts. Commercially prepared allografts are reported to contain BMP-2, BMP-4 and BMP-7, albeit at lower concentrations than from fresh bone preparations (Shigeyama et al, 1995). Allograft quality is also donor-dependent and variations in clinical outcome also depend upon the processing and handling methods of the allograft (Calori *et al*, 2011).

## **1.5.3.3** Bone Tissue Engineering (BTE)

To alleviate the shortcomings associated with traditional bone grafting techniques, the field of bone tissue engineering (BTE) is emerging, with the aim of providing suitable bone grafts substitutes. Scaffolds are regarded as an essential component for BTE as they are required to provide a porous, 3D environment, which permits the attachment and proliferation of cells, in addition to providing mechanical integrity to bone defects (Ma and Langer, 1999; Fröhlich *et al*, 2008).

As such, scaffolds that can mimic the natural ECM of bone are regarded as one of the most important challenges in successful tissue engineering (Vasita and Katti, 2006). Bone tissue constitutes around 10-30% protein by mass, with the remaining 70-90% comprising of calcium phosphate-based mineral, primarily hydroxyapatite (HAp) (Gokhale *et al*, 2001). Of the protein component of bone ECM, collagenous proteins constitute around 90% of the protein mass, of which around 90% is type I

collagen (Anselme, 2000; Young, 2003). The remaining 10% of bone protein is composed of non-collagenous proteins (NCPs), including OPN, BSP, OC and molecules containing arginine-glycine-aspartic acid (RGD) sequences, such as fibronectin and thrombospondins (Clarke, 2008). Additional NCPs include SLRPs, such as biglycan, decorin, perlecan and syndecan, which are able to bind osteoinductive growth factors (Lamoureux *et al*, 2007).

As the principle component of bone, type I collagen is regarded as a model candidate for a scaffold material (Aravamudhan *et al*, 2013), as it is characteristically biocompatible and biodegradable with the capacity to promote attachment, proliferation and differentiation of cells (Hiraoka *et al*, 2003). However, the mechanical properties of type I collagen are poor (Harley *et al*, 2007). HAp has also been widely studied as a bone-grafting substitute, owing to its excellent integration with host tissue (Bauer *et al*, 1991), high biocompatibility; and osteoinductive and osteoconductive properties, relating to its ability to bind and concentrate BMPs *in vivo* (LeGeros *et al*, 2002; Wei and Ma, 2004). However, HAp ceramics for bone tissue engineering are usually restricted to areas of low load bearing, due to its low mechanical strength (Zhou and Lee, 2011).

A number of commercially available bone grafting substitutes are currently available and have demonstrated positive results in augmentation of bone tissue. Studies analysing the efficacy of Bio-Oss<sup>®</sup>, comprised of deproteinised bovine bone, have yielded conflicting results. When placed into tooth extraction sockets, Bio-Oss<sup>®</sup> has demonstrated little bone growth accompanied with extensive connective tissue (Becker *et al*, 1998), in contrast to observed osseointegration and bone formation in another study, albeit with poor resorption (Artzi *et al*, 2000).

A range of other naturally derived substitutes for use in BTE have also been investigated, including chitosan (Venkatesan and Kim, 2010; Croisier and Jérôme, 2013) and silk-based matrices (Jones *et al*, 2009; Uchida *et al*, 2014). These naturally derived polymers are attractive candidates as bone grafting substitutes, as they demonstrate excellent biocompatibility and degradability (Freier *et al*, 2005; Yang *et al*, 2007a; Lu *et al*, 2011).

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Synthetic scaffolds are of additional interest in BTE, as they offer several advantages over biological scaffolds, such as lower cost, improved batch consistency and long shelf life (Dhandayuthapani *et al*, 2011). Synthetic polymers used for tissue engineering applications, include poly (glycolic acid), poly (lactic acid) and poly (propylene fumarate), which have been shown to be biocompatible and degradable (James *et al*, 1999; Wolfe *et al*, 2002; Liu and Ma, 2004; Gentile *et al*, 2014). Studies investigating the efficacy of these bone-grafting alternatives in humans are currently limited.

## 1.6 Dentine Matrix and Bioactivity

#### 1.6.1 Dentine Structure and Constituents

Dentine is a mineralised tissue present within mammalian teeth that resides between the outer layer of enamel and pulp chamber, with the main function of protecting the dental pulp. Dentine is secreted and maintained by odontoblasts, which reside on the boundary of the dentine and pulp; and project cell processes into tubules present within dentine (Arana-Chavez and Massa, 2004). These tubules are typically around 2-4 $\mu$ m in diameter and are present at an abundance of around 20,000/mm<sup>2</sup> (Schilke *et al*, 2000).

By weight, dentine is composed of 70% mineral, 20% organic protein and 10% water (Goldberg *et al*, 2012), similar to bone, which contains 60% mineral, 25% organic matrix and 15% water. Around 90% of the organic matrix of dentine and bone consists of type I collagen (Berkovitz *et al*, 2011), with the remaining 10% consisting of NCPs (Butler and Ritchie, 1995). These include BSP, dentine matrix protein 1 (DMP-1), dentine sialoprotein (DSP) and dentine phosphoprotein (DPP), with the latter two being characteristically found in dentine. These NCPs belong to a family of molecules called the small integrin-binding ligand N-linked glycoproteins (SIBLINGs), which contain RGD domains for which function as nucleating factors for the process of mineralization (Smith *et al*, 2012).

While presence of these ECM proteins has been long established, the vast proteome of dentine has only recently been extensively studied. The first comprehensive proteomic analysis of human dentine revealed 233 total proteins from three different patients, with sixty-eight of these proteins common between donors (Park *et al*, 2009). Chun *et al*, (2011) revealed 147 ethylenediamine tetraacetic acid (EDTA) soluble tooth proteins, which were confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography/mass spectroscopy (LC/MS). The extracted proteins exhibited differentiation enhancement of dental pulp-derived, stem cells (DPSCs), to produce mineral deposits when co-cultured in osteogenic differentiation medium. A later study revealed that extraction of human dentine by EDTA, followed by further extraction with guanidium hydrochloride, revealed a total of 289 different protein fractions (Jágr *et al*, 2012).

In addition to a range of matrix proteins, dentine also serves as a reservoir for a number of bioactive growth factors. TGF- $\beta_1$  has been identified as a pertinent growth factor in dentine with roles acknowledged for migration and proliferation of dental pulp-derived cells (Nie *et al*, 2006; Howard *et al*, 2010). Crucially, TGF- $\beta_1$  is implicated in odontoblast differentiation and subsequent matrix production in dentinogenesis (Smith et al, 1995; Tziafas and Papadimitriou, 1998). FGF-2 induces migration (Suzuki et al, 2011) and proliferation of dental pulp cells (He et al, 2008). PDGF enhances proliferation of fibroblasts in human dental pulp (Rutherford et al, 1992), in addition to stimulating expression of DSP in rat dental pulp cells (Yokose et al, 2004). VEGF serves to induce endothelial differentiation of human dental pulp cells (Marchionni et al, 2009) and has been shown to increase micro-vessel density in human severed dental pulps (Mullane et al, 2008). IGF-1 is proposed to drive differentiation of dental pulp cells (Joseph *et al*, 1993; Caviedes-Bucheli *et al*, 2007). BMPs possess potent effects on differentiation of dental pulp cells, notably BMP-2, BMP-4 and BMP-7 (Nakashima et al, 1994; Jepsen et al, 1997; Six et al, 2002; Saito et al, 2004).

## **1.6.2** Dentinogenesis

The process of tooth formation originates from the interaction of dental epithelial cells with underlying mesenchyme, leading to the formation of a bud. Once the bud has formed, the dental epithelium undergoes significant proliferation initially forming a cap which progresses to form a bell shaped structure (Duailibi *et al*,

2000). Within the formed invagination, differentiation of neural crest-derived cells into odontoblasts occurs (Ruch *et al*, 1995), which form small processes towards the enamel organ and subsequently become accentuated as the cell body of the odontoblast migrates towards the pulpal tissue. During migration, odontoblasts secrete large type III collagen fibrils, around 0.1-0.2µM in diameter, known as mantle dentine (Aguiar and Arana-Chavez, 2007; Goldberg *et al*, 2011). Fibronectin is also secreted and possesses a role in odontoblast polarisation (Lesot *et al*, 2001). Odontoblasts additionally synthesise and secrete a range of NCPs, including dentine sialophosphoprotein (DSPP) (Begue-Kirn *et al*, 1998), DMP-1 (Narayanan *et al*, 2006), biglycan and decorin (Goldberg *et al*, 2005). Mantle dentine calcifies at a mineralisation front as a result of the interactions between NCPs and hydroxyapatite crystals. CS-conjugated biglycan and decorin are closely associated with dentine calcification at the mineralisation front and are considered as pertinent components for regulation of dentine mineralisation (Embery *et al*, 2001; Waddington *et al*, 2003b; Milan *et al*, 2005).

Once the initial dentine has been formed, referred to a primary dentine, the odontoblasts retain their functional capacity and continue to form additional dentine, known as secondary dentine. This physiological process occurs throughout the life of the tooth, although at a reduced rate compared to primary dentine formation. Secondary dentine possesses a similar morphology to primary dentine with regards to the structure of the tubules (Aguiar and Arana-Chavez, 2007; Sloan and Smith, 2007).

## 1.6.3 Dentine Repair

Dentine has the capacity to be regenerated when damaged, with the production of a third type of dentine, known as tertiary dentine. Tertiary dentine can be further characterised into reactionary dentine and reparative dentine. Reactionary dentine is produced by odontoblasts that are responsible for the production of primary and secondary dentine. Mild stimuli, such as attrition, caries and the preparation of the tooth for a restoration, can cause surviving odontoblasts to deposit reactionary dentine to repair the tooth. The content of organic matrix and inorganic mineral are noted to be similar to that found in primary and secondary dentine (Smith *et al*, 1995; Arana-Chavez and Massa, 2004). Reparative dentine is laid down in response to a strong stimulus, such as deep caries. The stimulus is great enough to cause death of odontoblasts and a population of undifferentiated cells within the pulp differentiates into odontoblast-like cells, which secrete reparative dentine and preserves viability of the pulp (Smith *et al*, 1995). Morphologically, reparative dentine is generally different from reactionary dentine. Reparative dentine has been shown to contain cells entrapped within the matrix which was also devoid of dentinal tubules, thus resembling structure of bone. Furthermore, OPN has been detected within reparative dentine matrix, whereas OPN was not identified within the matrix of non-reparative dentine (Aguiar *et al*, 2007).

## 1.6.4 Osteogenic Potency of Dentine Matrix

One of the earliest recorded examples of exploring dentine as a grafting material for bone regeneration dates back to the 1960s, where it was acknowledged that rabbit dentine grafted into bone defect sites resulted in induction of bone formation, with demineralised dentine demonstrating greater induction ability than calcified dentine (Yeomans and Urist, 1967). A couple of decades later, dentine slices placed into inter-radicular osseous defects in dogs resulted in a 100% success rate for bone formation, with no bone resorption recorded (Catanzaro-Guimarães et al, 1986). More recently, several studies have further investigated the effectiveness of dentine matrix in stimulating the repair of bone tissue. Slices of demineralised dentine matrix (DDM) have been demonstrated to improve the volume of bone matrix compared to controls, when placed into mandibular defects in rabbits, whilst being resorbed during the bone remodelling process (Carvalho et al, 2004). DDM slices placed into surgical defects of diabetic rabbit parietal bone, using a polytetrafluoroethylene (PTFE) barrier have shown an increased radiopacity and improved trabecular bone arrangement, rivalled to non-treated rabbits and those treated with PTFE only (Gomes et al, 2008). Of late, it has been discovered that DDM placed into the tooth sockets of rats hastens the repair of the defect by improving the rate of new bone formation, in addition to enhancing the expression of VEGF compared to sockets treated with a blood clot control (Reis-Filho et al, 2012). Furthermore, human DDM placed into rat tooth sockets resulted in increased numbers of osteoblasts expressing BMP-2 and BMP-4, compared with unfilled sockets after 10 days (de Oliveira *et al*, 2013).

#### 1.6.5 Clinical Translation of Autologous Dentine for Bone Augmentation

Based on the well documented potential for dentine matrix to enhance bone regeneration, Kim et al (2010) proposed a method for preparation of autologous teeth to be used as a bone graft substitute in humans, referred to as an autologous bone-tooth graft (AutoBT). In the study, grafting material is prepared by extraction of teeth (usually for orthodontic reasons), followed by crushing into powder, dehydrating and sterilising with ethylene oxide. AutoBT has demonstrated bone healing capacity, in addition to augmented bone formation and remodelling when implanted into alveolar bone (Kim et al 2010). A more recent clinical trial has determined AutoBT as effective as a grafting material as Bio-Oss<sup>®</sup>, in addition to no reported incidences of infection or secondary complications (Pang et al, 2016). Despite the preliminarily promising results of AutoBT as a bone graft substitute, the procedure is time consuming and can take between days and weeks to prepare the graft material post-extraction (Binderman et al, 2014). In addition, there is a limited productivity for commercialisation due to required dehydration, which decreases shelf-life (Kim, 2015). Furthermore, whilst harvesting dentine from a single tooth provides sufficient volume of particulate to fill extraction sockets (Binderman et al, 2014), the aforementioned studies relating to the efficacy of autologous dentine for bone augmentation have only been used for applications relating to extraction sockets. Thus, the use of autologous dentine for bone repair may be limited to oral applications, as there is a deficit of studies relating to humans for the use of dentine as a bone graft for extra-oral sites. In addition, there are currently also very limited reports investigating the use of allogenic dentine as a bone-grafting substitute in human trials. However, in vivo studies in animals demonstrate positive bone forming capacity of allogenic dentine for enhancing bone repair (Al-Namnam et al, 2010). Due to the issues raised regarding dentine as a grafting material, determination of the components present within DDM that contribute towards osteogenesis may lead to the development of technologies that could result in clinically translatable BTE substitutes.

## **1.7** Aims and Objectives

The aim of this Thesis was to determine the potential for DDM in directing osteogenic responses of human BMMSCs and the cellular influences of DDM, relating to the process of bone repair through osteogenic differentiation. The presence and concentrations of a panel of growth factors, in addition to biglycan and decorin, within DDM, was examined and the significance of these factors for driving osteogenesis was determined. The potential for a biocompatible substrate containing demineralised dentine matrix for supporting osteogenesis was also assessed. These aims were achieved via the following:

- Characterisation of BMMSCs isolated, via adherence to fibronectin, for use with determining the osteogenic potential of DDM (Chapter 2)
- Determining the effects of DDM on cell expansion, apoptosis, migration and differentiation (Chapter 3)
- Elucidation of growth factor profile in DDM, via Western blot and quantification of their abundance by enzyme-linked immunosorbent assays (ELISAs). Depletion of growth factors from DDM via heparin-affinity chromatography and assessment of the osteogenic induction potential of fractionated-DDM (Chapter 4)
- Confirmation of presence of the SLRPs, biglycan and decorin, in DDM, depletion via immunoprecipitation and assessment of the osteogenic induction potential of SLRP fractionated DDM (Chapter 5)
  - Preliminary analysis of silk-fibroin/gelatin (SF/G) based substrates loaded with DDM for supporting osteogenesis of BMMSCs (Chapter 6)

It is hypothesised that within the vast proteome of dentine, selected constituents may be imperative for its osteogenic properties, which can be identified as possessing roles for directing bioactivity towards BMMSCs.

# Chapter 2 - Selection and Characterisation of Human Bone Marrow-Derived, Mesenchymal Stem Cells (BMMSCs)

## 2.1 Introduction

Over recent years, there has been a marked increase in interest in the study of mesenchymal stem cells (MSCs) for their use in tissue engineering and regenerative medicine. Friedenstein *et al*, (1970) originally identified MSCs from bone marrow, and since then many other tissue sources of MSCs have been identified, including, but not limited to, umbilical cord blood (Mareshci *et al*, 2001), adipose tissue (Zuk *et al*, 2001), dental pulp (Gronthos *et al*, 2000) and articular cartilage (Alsalameh *et al*, 2004). Amongst MSC sources, the efficacy for bone marrow-derived, mesenchymal stem cells (BMMSCs) in stimulating bone repair has been widely studied with promising results (Fang *et al*, 2007; Mankani *et al*, 2007; Brennan *et al*, 2014).

Due to variations of tissue source, methods of isolation and culture, criteria were developed to aid in the standardisation of MSCs to avoid discrepancies between laboratories. Criteria relating to surface adherence, cell surface marker expression and differentiation potential have been outlined to assist in characterisations of MSCs. Amongst others, cells must demonstrate positive expression for CD105, CD90 and CD73, as well as negative expression for CD34 and CD45 (Dominici *et al*, 2006). Embryonic markers of pluripotency, such as Oct4 and Nanog, are additionally proposed as candidates for determining undifferentiated MSCs *in vitro* (Tsai *et al*, 2012). Although various additional markers have been suggested, no definitive marker is solely specific for determining MSC phenotype (Feng-Juan *et al*, 2014).

MSCs exist as a heterogeneous population, consisting of clonal and non-clonal cells. Analyses of clonal MSCs demonstrate a hierarchy of lineage commitment, by which tri-potent progenitors yield more restricted bi-potent cells. These bi-potent cells subsequently give rise to slow growing precursors of differentiation potential to a single lineage (Phinney, 2012). As a consequence of the existence of a differentiation hierarchy, clonal populations of BMMSCs demonstrate variability with regards to differentiation potential, by sequential loss of adipogenic and chondrogenic potential, to produce osteoprogenitors (Muraglia *et al*, 2000; Russell *et al*, 2011). Furthermore, osteogenic potential of BMMSCs is reported to vary between donors (Phinney *et al*, 1999; Mendes *et al*, 2002).

The existence of a lineage hierarchy and resulting variations in differentiation potential of BMMSCs results in inconsistencies with regard to therapeutic efficacy (Siddappa *et al*,2007; Prins *et al*,2009). Primitive cells express high levels of  $\alpha$ 5 $\beta$ 1 integrin (Jones and Watt, 1993); and can be isolated via rapid adhesion to fibronectin (Wu *et al*, 1993), thus providing a method for isolating a sub-population of undifferentiated cells from heterogeneous cell preparations. Fibronectin adherence of MSCs has demonstrated isolation of primitive MSCs from bone marrow and dental pulp (Waddington *et al*, 2009; Harrington *et al*, 2014). Undifferentiated MSCs possess the ability to proliferative and subsequently differentiate into osteoblasts that deposit reparative bone matrix and thus, results in fracture repair (Tseng *et al*, 2008; Knight and Hankenson, 2013).

This Chapter aimed to characterise commercially available MSCs from bone marrow origin, with regards to expansive capacity and gene expression relating to a range of cell surface markers to confirm MSC phenotype. Isolation of a fibronectin-adherent (FNA) sub-population was performed with marker expression and expansive capacity characterised and compared to unselected cells. Pertinently, the purpose of this Chapter was to identify a population with greater homogeny for primitive BMMSCs that were used for all forthcoming experimental procedures within this Thesis.

## 2.2 Materials and Methods

## 2.2.1 Cell Source

Human BMMSCs were obtained commercially (Lonza, UK). BMMSCs were certified to be >90% positive for CD105, CD166, CD29 and CD44 and <10% positive for CD14, CD34 and CD45. Donation of BMMSCs was from a 30-year-old male.

# 2.2.1.1 Culture Expansion of BMMSCs in vitro

All cells were cultured in Minimum Essential Media, Alpha Modifications (α-MEM), containing ribonucleosides and deoxyribinucleosides (Gibco, Thermo Fisher Scientific, USA). Medium was supplemented with 10% foetal bovine serum (FBS) (Invitrogen, UK), 100µM L-ascorbic-2-phosphate (Sigma-Aldrich, UK), 100 units/mL penicillin, 0.1µg/mL streptomycin and 0.25µg/mL amphotericin B (Antibiotic/Antimycotic, Sigma-Aldrich).

Cryopreserved cells were thawed by mixing with 5mL of media, warmed to  $37^{\circ}$ C and then centrifuging at 450g for 5 min. Supernatant was aspirated and the remaining cell pellet re-suspended in 5mL of media. Cell solution was centrifuged at 450g for 5 min, supernatant aspirated and cell pellet re-suspended in 1mL of media. Cell numbers were determined by mixing  $10\mu$ L of the resulting suspension in a 1:1 ratio with a 0.4% Trypan Blue solution (Sigma-Aldrich); counted in a Bürker haemocytometer. Cell viability was determined by assessing the ratio of live cells relative to total cells counted. Cells were cultured in vented culture flasks or culture dishes (Sarstedt, UK),  $37^{\circ}$ C, 5% CO<sub>2</sub>, at a seeding density of  $5x10^{3}$  cells/cm<sup>2</sup>.

At 70-80% confluence, culture medium was aspirated and cells were briefly washed with Phosphate Buffered Saline (PBS). Cells were treated with Accutase (PAA, Austria), at 37°C, until all cells had detached from the culture surface. Accutase was neutralised by adding an equal volume of serum-containing culture medium and pipetting up and rinsing down the culture surface 10 times pooled all of the cells. The Accutase/medium solution was aspirated from the culture flask and centrifuged at 450*g* for 5 min to pellet cells. The supernatant was decanted and cells were re-suspended in 1mL of culture medium by pipetting up and down 30 times.

## 2.2.1.2 Fibronectin-adherence Selection of BMMSCs

Fibronectin from human plasma (Sigma-Aldrich) was reconstituted in PBS, supplemented with 1mM calcium chloride (CaCl<sub>2</sub>) (Sigma-Aldrich) and 1mM magnesium chloride (MgCl<sub>2</sub>) (Sigma-Aldrich) (PBS<sup>++</sup>), to a final concentration of 10µg/mL. 1mL of this solution was plated into individual wells of two 6-well plates (Sarstedt), wrapped in Parafilm (Bemis, USA) and stored overnight at 4°C. The Fibronectin/PBS<sup>++</sup> solution was aspirated and BMMSCs of 5.9 population doublings (PDs) were seeded onto the fibronectin-coated plates, at a density of 4x10<sup>3</sup> cells/cm<sup>2</sup>. The plates were incubated at 37°C at 5% CO<sub>2</sub> for 20 min. Cell culture medium was aspirated, replaced with fresh medium and cells returned to incubation. FNA BMMSCs were cultured and propagated throughout expansive culture, as described previously (Section 2.2.1.1).

## 2.2.1.3 Determination of Population Doublings (PDs)

Cell numbers were determined, as described previously (Section 2.2.1.1). Cell counts were used to determine PDs, proportional to the number of cells seeded initially, using the equation:

PDs

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

Cells were reseeded at  $4x10^{3}$ /cm<sup>2</sup>. Remaining cells were centrifuged at 450g for 5 min, re-suspended in 1mL of freezing media (Bambanker, Wako, USA) at densities of  $5x10^{5}$ - $2x10^{6}$  cells/mL and stored at -196°C. Reseeded cells were propagated throughout expansive culture to obtain stocks of each passage. Cells were tested for contamination by Mycoplasma every 2 weeks and were treated with BM Cyclin treatment (Roche, Germany), for 2 weeks if found positive for Mycoplasma contamination. Cells were tested for Mycoplasma again and were cultured as normal if found to be negative, or discarded if still tested positive.

## 2.2.2 β-galactosidase Stain for Cellular Senescence

BMMSCs/FNA BMMSCs were cultured to suspected senescence as indicated by expansive plateau (15.2 PDs BMMSCs, 21.3 PDs FNA BMMSCs) and morphological appearance. Cells were passaged and seeded into 3 wells of a 6-well plate. Cells from pre-senescent cultures (12.7 PDs BMMSCs, 17.6 PDs FNA BMMSCs) were also plated in an identical fashion. β-galactosidase staining was performed using a Senescent Cells Histochemical Staining Kit (Sigma-Aldrich), following the manufacturers protocol. Two days after plating, culture media was aspirated and cells were washed twice with 1mL of PBS per well, ensuring thorough removal of residual fluid. Fixation Buffer was diluted to a 1x working concentration, 1.5mL applied to each well and incubated at room temperature for 6-7 min. The plate was rinsed thrice with 1mL of PBS per well, followed by addition of 1mL per well of preprepared Staining Solution. Culture plates were sealed with Parafilm (Bemis) to prevent drying out and were incubated at 37°C overnight to enhance staining. Five random and independent areas per well were photographed under light microscopy, using a Panasonic DMC-G1 Camera.

## 2.2.3 RNA Extraction for Analysis of Gene Expression

BMMSCs and FNA BMMSCs of earlier and later culture points were seeded at  $4 \times 10^3$  cells/cm<sup>2</sup> in 3 wells of a 6-well plate and cultured until 90-100% confluent. RNA was extracted using an RNeasy kit (Qiagen, Germany), following manufacturer protocol. All centrifugation steps were performed at 17,000*g* at ambient temperature.

Culture media was aspirated and each well was washed with 600µL of PBS. Cells were lysed using 600µl of RLT Lysis Buffer supplemented with 1% 2-mercaptoehtanol (Sigma-Aldrich), with further lysis performed by transfer of the cell lysate to a QIAshredder<sup>™</sup> tube and centrifuging for 2 min.

Shredded cell lysate was mixed with an equal volume of 70% (v/v) ethanol (Sigma-Aldrich), 600 $\mu$ L transferred to an RNeasy Mini Kit column and centrifuged for 15 s. Flow through was discarded and remaining cell lysate solution applied through the column. The column was washed with 350 $\mu$ L of RW1 Buffer by centrifuging for 2 min. The flow through was discarded and 10 $\mu$ L of RNase-free DNase was mixed with 70µL of RDD Buffer and applied to the column membrane for 15 min. Columns were washed with 350µL of RW1 Buffer by centrifuging for 15 s. Two further column washes were performed with 500µL of RPE Buffer, centrifuging for 15 s for the first wash and 2 min for the second wash, with flow through discarded after each wash. The columns were transferred to sterile RNase-free 1.5mL micro centrifuge tubes (Eppendorf, UK); and 30µL of RNase-free water was added to the column and centrifuged for 2 min to yield RNA.

Total RNA concentration was measured using a NanoVue spectrophotometer (GE Healthcare, UK), calibrated with  $2\mu$ L of RNase-free water.  $2\mu$ L of RNA solution was used for RNA quantification. A260/A280 ratio was additionally recorded with A260/A280 ratio of >1.7 judged acceptable for purity of RNA.

## 2.2.4 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

All reagents used for reverse transcription-polymerase chain reaction (RT-PCR) were from Promega (UK). 1µg of extracted RNA was mixed with 1µL of Random Primers and made up to a total volume with nuclease-free water of 15µL, in a sterilised 0.2mL tube (Eppendorf). 1µg of total human RNA (Clontech, France) was additionally converted to cDNA in an identical manner to cell extract RNA, as a positive control for markers anticipated to not be expressed. A reverse transcription (RT) negative was generated using nuclease-free water in place of RNA. Reagents were incubated at 70°C for 5 min in a Thermal Cycler (G-Storm, UK), before being immediately cooled and stored on ice.

A master mix was prepared by adding 5 $\mu$ L of 5x MMLV Reaction Buffer, 1.25 $\mu$ L of 10mM dNTPs, 0.6 $\mu$ L of RNasin, 1 $\mu$ L of MMLV enzyme and 2.15 $\mu$ L of nuclease-free water, in a sterilised 1.5mL tube (Eppendorf). 10 $\mu$ L of master mix was transferred to the 15 $\mu$ L of RNA/Random Primer mix before incubating at 37°C for 1 h. Resulting cDNA was stored at -20°C.

## 2.2.4.1 Polymerase chain Reaction (PCR) cycling

For each gene to be analysed (as shown in Table 2.1), a master mix consisting of  $5\mu$ L of 5X Buffer, 0.5 $\mu$ L of 10mM dNTPs, 1.25 $\mu$ L of 3mM for each primer (forward and reverse), 1 $\mu$ L of 25mM MgCl<sub>2</sub> (1mM final concentration), 0.25 $\mu$ L of Taq Polymerase

and 14.75µL of nuclease-free water, was prepared in a sterile 0.2mL tube (Eppendorf). 1µL of cDNA/RT negative was added to the master mix. 1µL of nuclease-free water served as a negative control for the PCR cycling. The sample was placed in a Thermal Cycler (G-Storm) and cycled as follows: one initial denaturation at 95°C for 4 min, thirty-five cycles of annealing and extension at 95°C for 1 min, annealing temperature for 1 min and 72° for 1 min; and one final cycle extension at 72° for 10 min. Samples were stored at 4°C, prior to gel visualisation. GAPDH was used as an internal reference gene.

#### 2.2.4.2 Agarose Gel Electrophoresis of PCR Products

A 2% agarose gel was prepared by adding 1.4g of Hi-Res Agarose powder (AGTC Bioproducts, USA) to 70mL of 0.5x tris/borate/EDTA (TBE) buffer (89mM Trizma Base, Sigma-Aldrich, 89mM boric acid, Sigma-Aldrich, and 2mM ethylenediamine tetra-acetic acid (EDTA), Sigma-Aldrich), in a sterilised beaker. The solution was heated in a microwave until the agarose had dissolved. The solution was cooled slightly, prior to adding 7µL of Safeview DNA stain (NBS Biologicals, UK) and mixing. The mixture was poured into an electrophoresis cast tray, assembled with a well comb and allowed to set at ambient temperature. The gel was transferred to an electrophoresis tank containing 0.5x TBE. 5µL of 100bp DNA ladder (Promega) was added to the first well and 10µL of PCR samples for analysis were added to subsequent wells. The samples were separated at 90V for approximately 40 min, or until the sample dye had run to approximately  $\frac{3}{4}$  the length of the gel. Band visualisation was performed using a Benchtop UV Transilluminator (UVP, Canada), with resulting images recorded.

Gene	Primer sequence: 5'-3'	Annealing	Product	Primer
product		temperature	length	designer
		(°C)	(bp)	
GAPDH	F: TTCTTTTGCGTCGCCAGCCGA	55	96	Author
	R: GTGACCAGGCGCCCAATACGA			
CD105	F: GAAACAGTCCATTGTGACCTTCAG	55	344	Dr. Amr
	R: GATGGCAGCTCTGTGGTGTTGACC			Alraies
				(Cardiff
				University)
CD90	F: ATGAACCTGGCCATCAGCATCG	55	425	Dr. Amr
	R: CACGAGGTGTTCTGAGCCAGCA			Alraies
				(Cardiff
				University)
CD73	F: GTCGCGAACTTGCGCCTGGCCGCCAAG	55	352	Dr. Amr
	R: TGCAGCGGCTGGCGTTGACGCACTTGC			Alraies
				(Cardiff
				University)
CD146	F: TGTGTAGGGAGGAACGGGTA	55	101	Author
	R: TGGGACGACTGAATGTGGAC			
CD34	F: GCTTTGCTTGCTGAGTTTGC	55	180	Author
	R: CCATGTTGAGACACAGGGTGC			
CD45	F: CCCCACTGATGCCTACCTTA	55	238	Author
	R: ATGCACCTCATTGTTTGTGC			
Oct4	F: GGTGGAGAGCAACTCCGAT	55	246	Author
	R: ATGGTCGTTTGGCTGAATACC			
Nanog	F: AGGTCCCGGTCAAGAAACAG	55	235	Author
	R: CCCTGCGTCACACCATTG			

Table 2.1 - Primer sequences used for identification of gene expression for MSC markers.

# 2.3 Results

# 2.3.1 Culture Expansion of BMMSCs

Upon acquisition, BMMSCs demonstrated 85% viability (indicated by Trypan Blue staining) when thawed and readily expanded in culture. Expansive culture was prolonged for around fifty-five days and ten passages (15.2 PDs), at which point BMMSCs demonstrated arrest in expansive potential. Time between passages was around five days up to passage seven (13.1 PDs), which increased to around seven days up until the tenth passage (Figure 2.1).

BMMSCs of passage three (5.9 PDs) were plated out and isolated via adhesion to fibronectin. Adherent cells (FNA BMMSCs) reached passage confluence after four days and propagated in expansive culture to 21.3 PDs (thirteen passages) after which expansive capacity declined (20.4 PDs at passage fourteen). Time between passages was around four to five days until passage eleven (19.1 PDs), where time between passages increased, as with BMMSCs, to around seven days (Figure 2.1).



Figure 2.1 - Population doubling level of BMMSCs (as acquired) and FNA BMMSCs. Nonselected BMMSCs undergo around 15.2 PDs prior to reaching expansive plateau, whereas FNA BMMSCs achieve 21.3 PDs before expansion declines.

# 2.3.2 Morphological Appearance Throughout Expansive Culture

At earlier culture of BMMSCs (3.9 PDs), morphology is typical of MSC cultures indicated by small, elongated and fibroblast-like appearance. At later culture (12.7 PDs), cells lose their definitive elongated shape and appear larger with stress-related fibres becoming apparent. At latest culture (15.1 PDs), cells completely lose fibroblast-like shape and become very large and flattened, accompanied with marked cytoplasmic accumulations (Figure 2.2).

FNA BMMSCs at earlier culture (10.5 PDs) demonstrated elongated, fibroblast-like shape, which is maintained through to later culture (17.6 PDs), with no discernible change in morphological appearance. Latest culture (20.4 PDs) demonstrated morphological changes akin to latest culture BMMSCs, characterised by loss of spindle shape, increased cell size and cytoplasmic debris (Figure 2.2).



Figure 2.2 - Representative micrograph images of BMMSCs and FNA BMMSCs during expansive culture at earlier, later and latest time-points. BMMSCs and FNA BMMSCs demonstrated spindle-shaped morphology at earlier points, which was maintained in FNA BMMSCs in later culture; however, BMMSCs began to appear larger. Both cell populations exhibited cell flattening and increased cytoplasmic aggregations (yellow arrows) at the latest culture points. White bars represent 100µM.

# 2.3.3 β-galactosidase Staining for Senescence Analysis

Cultures of both BMMSCs (15.1 PDs) and FNA BMMSCs (20.4 PDs) were obtained and assessed for  $\beta$ -galactosidase staining to assess the senescent nature of the cell population. In Figure 2.3, both cell populations were shown to contain a large proportion of cells that had blue staining evident within distinct regions of the cell cytoplasm. However, not all cells demonstrated positive staining for  $\beta$ galactosidase, despite demonstrating morphological appearances similar to that of positively stained cells. Both cell populations were additionally stained for  $\beta$ galactosidase enzyme activity at pre-senescent cultures (BMMSCs 12.7 PDs, FNA BMMSCs 17.6 PDs), with positive staining only present in a very small proportion of cells (Figure 2.3).



Cell population	Cells positively stained for β-galactosidase (%)
BMMSCs (Late culture, 12.7 PDs)	3.0%
BMMSCs (Latest culture, 15.1 PDs)	58.7%
FNA BMMSCs (Late culture, 17.6 PDs)	2.4%
FNA BMMSCs (Latest culture, 20.4 PDs)	75.0%

Figure 2.3 - Micrographs of BMMSCs stained for  $\beta$ -galactosidase enzyme activity at presenescent (left) and senescent (right) culture and percentage of positively stained cells. BMMSCs in pre-senescent culture appear smaller and less than 5% stain for  $\beta$ -galactosidase (black arrows). Conversely, cells in later culture appear to possess larger, flattened morphology concurrent with prominent (>50%) blue staining (white arrow heads). White bars represent 100µM.

## 2.3.4 Mesenchymal stem cell (MSC) Marker Expression

A panel of different markers were assessed by PCR for characterisation of earlier (3.9 PDs BMMSCs/10.5 PDs FNA BMMSCs), and later culture (12.7 PDs BMMSCs/ 17.6 PDs FNA BMMSCs) BMMSCs and FNA BMMSCs. Both cell populations expressed the mesenchymal markers, CD105, CD90 and CD73, up to late culture, in addition to CD146. CD34 and CD45 displayed very low expression levels in both cell populations at both earlier and later culture. Embryonic markers, Oct4 and Nanog, were positively expressed in both cell populations with maintained expression throughout culture. Osteogenic commitment marker RunX2 was not expressed in BMMSCs, however very weak expression is apparent in FNA BMMSCs at later culture (Figure 2.4). No bands were detected in RT negative or PCR negative reactions. CD34, CD45 and RunX2 demonstrated prominent expression in DNA obtained from total human RNA, thus indicating validity of primers.





#### 2.4 Discussion

Heterogeneity in BMMSC populations is well acknowledged and results in inconsistencies in differentiation potential between cell populations. Experimental procedures within this Chapter were performed to acquire and characterise a population of more refined cells from a heterogeneous population, via adhesion to fibronectin. FNA BMMSCs maintained MSC marker expression throughout culture in addition to an expansive capacity around 33% greater than whole BMMSCs, prior to reaching senescence. FNA BMMSCs thus represent a refined population of characterised, immature cells with which cell behaviour has been assessed in subsequent Chapters.

Initial acquisition of BMMSCs was from a commercially available source and the cells provided were certificated to conform to MSC characteristics with regards to tri-lineage differentiation potency and marker expression, including CD105<sup>+</sup>, CD90<sup>+</sup>, CD73<sup>+</sup>, CD34<sup>-</sup> and CD45<sup>-</sup>. Even though the term MSC is used to define cells adhering to the International Society for Cellular Therapy (ISCT) guidelines (Dominici *et al*, 2006), mounting reports highlight the heterogeneity of MSCs with regard to these defining criteria. Heterogeneity of MSCs can simply been noted via micro-graphical observations of MSCs, with three observed phenotypes noted; spindle-shaped, star-shaped and large/flat cells (Muraglia *et al*, 2000; Xiao *et al*, 2010).

Due to the reported heterogeneity of MSCs, adherence of BMMSCs to fibronectin was utilised to purify a more immature sub-population of cells. Fibronectin serves as a ligand to cells expressing  $\alpha_5\beta_1$  integrin (Wu *et al*, 1993). Cell surface expression of  $\beta_1$  integrin and rapid adhesion to extracellular matrix (ECM) molecules correlates with proliferative capacity and high colony forming efficiency, with a two-fold increase of surface  $\beta_1$  integrin expression present on stem cells, compared to transit amplifying cells (Jones and Watt, 1993; Jones *et al*, 1995). Consequently, primitive cells with high  $\beta_1$  integrin expression can be isolated via adherence to fibronectin (Jones *et al*, 1995; Dowthwaite *et al*, 2004, Waddington *et al*, 2009). Isolation of FNA cells derived from bone marrow has yielded cells positive for CD105, CD90 and CD73 with negative CD34 expression, along with high and low proliferative potential (Xiao *et al*, 2010). Populations of FNA MSCs derived from bone marrow display heterogeneity with regards to proliferative potential and population doubling level. However, with differentiation potential towards osteogenic, chondrogenic and adipogenic lineages present in all cells isolated via fibronectinadherence (Harrington *et al*, 2014).

During expansive culture, BMMSCs achieved 15.2 PDs prior to reaching a plateau of cell doubling, however, fibronectin isolation of BMMSCs was successfully achieved in this study and yielded a population of FNA BMMSCs with expansive capacity in vitro of 21.3 PDs, prior to expansive decline. Various literature studies report discrepancies between the expansive capacities of BMMSCs derived from human donors. Wagner et al, (2008) analysed six donor populations of BMMSCs and reported cumulative PDs ranging from between six to sixteen prior to reaching growth arrest, in addition with an estimated additional seven to nine PDs during initial colony formation. Ren et al, (2013) reported PDs of BMMSCs from seven donors to vary from twenty-five to forty before expansive capacity was achieved. Although BMMSCs used in this Thesis underwent fewer PDs compared to those analysed within literature reports, they were received at passage two with no receipted reference to PDs from initial isolation. Therefore, expansive capacity from original isolation would have been greater than indicated. It is additionally important to note that discrepancies between observed expansive capacities of human BMMSC preparations can be attributable to specific methods of isolation and culture (Siddappa *et al*, 2007; Ho *et al*, 2008).

Studies involving the characterisation of MSCs *in vitro* have extensively utilised expression of cell surface markers to determine cellular phenotype. As no single marker for determining MSCs has been identified to date, assessing the expression of a panel of diverse markers is regularly utilised to determine MSC phenotype *in vitro*. CD105 is highly expressed in human MSC preparations (Schieker *et al*, 2007), with over 95% of MSCs expressing CD105 (Lodie *et al*, 2002). CD90 is indicated as a universally expressed marker for MSCs that maintains expression throughout MSC *in vitro* culture (Musina *et al*, 2005; da Silva Meirelles *et al*, 2006). Additionally, CD73 expression has been affiliated with MSCs (Haynesworth *et al*, 1992; Etheridge *et al*, 2004). Co-current with ISCT guidelines (Dominici *et al*, 2006), BMMSCs and

FNA BMMSCs expressed mRNA for CD105, CD90 and CD73 throughout expansive culture. However, the positive expression of CD105, CD90 and CD73 has also been affiliated in fibroblasts and endothelial cells (Narravula *et al*, 2000; Alt *et al*, 2011); and therefore the expression of these markers alone are not specific for MSCs.

Literary reports demonstrate a general consensus regarding negative expression of endothelial cell markers, CD34 and CD45, within MSC preparations (Pittenger et al, 1999; Zuk et al, 2002; Dominici et al, 2006; Saccheti et al, 2007). Regarding results presented in this Thesis, PCR analyses of BMMSCs and FNA BMMSCs indicated that relative to other markers, CD34 and CD45 mRNA expression in both cell types was shown to be very low, although not entirely negative. Kaiser et al (2007) reported that a small proportion of isolated MSCs positively express CD34 and CD45. In addition, MSCs from populations both negative and positive for both CD34 and potency. CD45 demonstrate adipogenic and osteogenic differentiation Furthermore, freshly isolated and uncultured MSCs from bone marrow origin demonstrate positive expression for CD34 (Simmons and Torok-Storb, 1991). Consequently, the presence, albeit small, of MSCs expressing haematopoietic surface markers is believed to result as a consequence of cell culturing (Lin et al, 2012).

Due to increasing awareness of other markers believed to be expressed in MSCs, additional analyses were undertaken in attempt to further characterise and compare BMMSCs with their FNA counterparts. CD146 is a transmembrane glycoprotein constitutively expressed in human endothelial cells (Bardin *et al*, 2001), with expression also observed in cells of smooth muscle, melanoma and follicular dendrite origin (Bardin *et al*, 1996). More recently, CD146 has been associated with expression in MSCs from bone marrow origin (Shi and Gronthos, 2003). CD146<sup>+</sup> MSCs have been demonstrated to be able to differentiate into osteoblasts (Saccheti *et al*, 2007), chondrocytes and adipocytes (Baksh *et al*, 2007), in addition to possessing extended proliferation during *in vitro* culture compared to CD146<sup>-</sup> MSCs (Sorrentino *et al*, 2008). Moreover, CD146 has been proposed as a marker of cell pluripotency, with clonal populations expression around 2-fold higher

for tri-lineage potential clonal populations, compared to uni-potent clones (Russell *et al*, 2010).

In this Chapter, Oct4 and Nanog were indicated to be expressed in both BMMSCs and FNA BMMSCs at earlier and later cultures. Although Oct-4 is characteristic of ESCs, expression has been identified in MSCs of bone marrow origin (Greco et al, 2007; Reikstina et al, 2009). Increases in Oct4 expression in MSCs has been demonstrated to increase differentiation efficiency towards osteogenic and adipogenic lineages (Roche et al, 2007). Nanog is reported to not be expressed in freshly isolated MSCs. However, expression is identified after in vitro culture in MSCs not induced to differentiate (Pierantozzi et al, 2011). Forced expression of Nanog in BMMSCs encourages maintenance of expansion (Go et al, 2008). Ectopic overexpression of Oct4 and Nanog in MSCs enhances proliferation and differentiation potential in culture, however, knock-down of Oct4 and Nanog reverses these observations (Tsai et al, 2012). Expression of Oct4 and Nanog is terminated in MSCs differentiated into osteoblasts, however, is maintained in MSCs prior to differentiation (Arpornmaeklong et al, 2009). Although Oct4 expression appears to be saturated for BMMSCs and FNA BMMSCs at earlier and later culture periods, Nanog expression appears to be greater for FNA BMMSCs, compared with BMMSCs. Resultantly, the perceived increase in Nanog expression supports the more immature and less heterogenic nature of FNA BMMSCs, compared to unselected BMMSCs.

In addition to analysing expression of mesenchymal and embryonic markers, expression of RunX2 was also investigated to further characterise BMMSCs and FNA BMMSCs throughout expansive culture. BMMSCs at earlier and later PDs did not demonstrate positive mRNA expression for RunX2, whereas FNA BMMSCs were negative for RunX2 expression at earlier PDs, however weak expression was identified at later PDs. RunX2 is widely regarded as the critical regulator in the initiation of osteoblastic differentiation (Otto *et al*, 1997; Sadhakar *et al*, 2001; Cohen, 2013). RunX2 is strongly expressed in immature osteoblasts. However, expression is down regulated during osteoblastic development and maturation (Maruyama *et al*, 2007). Expression of RunX2 is, therefore, associated with directing

multipotent MSCs towards the osteoblastic lineage (Komori, 2010) and expression also results in inhibition of differentiation of MSCs to adipocytes and chondrocytes (Komori, 2006). The observed expression of RunX2 in addition to maintained expression of other mesenchymal markers at later PDs of FNA BMMSCs indicates a change in profile of cells regarding population homogeneity. RunX2 expression indicates the lineage commitment of a proportion of FNA BMMSCs towards the osteoblastic lineage. Thus, any cells beyond this number of PDs did not constitute a purified population of primitive cells and were not utilised for upcoming experimental procedures within this Thesis.

With prolonged culture, MSCs enter a senescent state and undergo phenotypic changes, including broadening and flattening of cells, accumulation of stress fibres, granular cytoplasm and increases in cellular debris (Bruder et al, 1997; Wagner et al, 2008). The point of growth arrest of both BMMSCs and FNA BMMSCs demonstrated these observations, which were not present during earlier culture periods. In addition, FNA BMMSCs did not exhibit these senescence-associated phenotypic observations at a comparable population doubling level to which BMMSCs appeared to be senescent, further indicating that FNA BMMSCs are at a more immature state than unselected BMMSCs.  $\beta$ -galactosidase is reported to only be active in senescent cells, however, it is not active in differentiated, quiescent or pre-senescent cells (Dimri et al, 1995); and is postulated to be as a result of increasing lysosomal mass during prolonged cellular replication (Lee et al, 2006). βgalactosidase activity is MSCs can be used as an additional measure of senescent state. β-galactosidase staining was performed on both populations of cells at the stage of suspected senescence, with positive staining observed in a large proportion of cells, however, not all senescence-phenotype cells stained positively. βgalactosidase assays are reported to predominantly stain large senescent cells and thus, is not an absolute determinant of senescence (Wagner et al, 2008; Zhou et al, 2008). Hence β-galactosidase staining was used in conjunction with morphological observations and expansion data to determine cellular senescence. Collectively, senescence of BMMSCs and FNA BMMSCs was determined to be reached after 15.1

PDs and 20.4 PDs, respectively, indicating the extended expansive capacity of FNA BMMSCs compared to BMMSCs.

Overall, results from this Chapter have demonstrated the isolation of a primitive population of cells from a heterogeneous population via fibronectin-adherence selection. The cells obtained possess greater expansive capacity *in vitro* compared to unselected cells with a prolonged culture period senescence and also demonstrate expression of a range of markers used to characterise MSCs. Thus, the FNA BMMSCs isolated present a purified population with less heterogeneity compared with unselected BMMSCs, and were utilised for all forthcoming experiments in this Thesis (here on in referred to as BMMSCs).

Chapter 3 - Characterisation of Biological Effects of Demineralised Dentine Matrix (DDM) on Bone Marrow-Dervied, Mesenchymal Stem Cells (BMMSCs)

## 3.1 Introduction

Bone has a high capacity for in vivo regeneration, attributable to the presence of a tightly regulated signalling environment and associated factors, including inflammatory cytokines (Einhorn et al, 1995; Rundle et al, 2006), growth factors (Bostrom et al, 1995; Cho et al, 2002; Hughes et al, 2006) and mesenchymal stem cells (MSCs) (Nakahara et al, 1990; Einhorn, 1998). However, delayed union or nonunion of fractures occurs in 5-10% of cases of bone trauma (Gómez-Barrena et al, 2015). Moreover, bone regeneration is hindered in several clinical situations, including osteoporosis (Nikolaou et al. 2009), diabetes mellitus (Loder, 1988); and as a consequence of ageing (Gruber et al, 2006). Such conditions compromise intrinsic bone healing and require intervention to achieve adequate tissue repair. To accomplish sufficient regeneration of large bone volumes, autologous bone grafts (ABGs) are required and although their rates of success are high, post-operative complications negate their use (Brugnami et al, 2009). Bone allografts provide an alternative source of tissue to augment tissue repair, however, success rates are much more variable due to batch variations and methods of processing (Calori et al, 2011).

Like bone, dentine is acknowledged to consist of a plethora of bioactive constituents, including growth factors (Finkelman *et al*, 1990; Roberts-Clark and Smith, 2000), inflammatory molecules (Graham *et al*, 2007) and extracellular matrix () proteins (Embery *et al*, 2001), all with proposed roles in orchestrating tissue repair responses. Sequestration of factors within the matrix is proposed to act as a 'reservoir' of signalling molecules (Tziafas *et al*, 2000; Goldberg *et al*, 2004). Carious injury to dentine results in demineralisation (Hojo *et al*, 1994; Larmas *et al*, 2001); subsequently sequestered factors are released and are proposed to contribute towards reparative dentinogenesis (Smith *et al*, 1995; Cooper *et al*, 2010). Clinically

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induced release of bioactive factors from dentine using ethylenediamine tetraacetic acid (EDTA), calcium hydroxide (CaOH<sub>2</sub>) and mineral trioxide aggregate (MTA) provides successful pulp capping, by inducing reparative responses of the dentine-pulp complex (Duque *et al*, 2006; Graham *et al*, 2006; Tomson *et al*, 2007).

The bioactivity of dentine for augmenting bone repair has been recognised *in vivo*, with demineralised dentine matrix (DDM) acknowledged to stimulate reparative responses in rat tooth sockets (Reis-Filho *et al*, 2012) and diabetic rabbit bone defects (Gomes *et al*, 2008). Whilst postulated that DDM can emulate the signalling environment required for bone repair, characterisation of bioactivity towards bone marrow-derived, mesenchymal stem cells (BMMSCs) is less well acknowledged. However, the potential for dentine matrix to induce osteogenic differentiation of BMMSCs has been noted in heterogeneous BMMSCs derived from rats (Yu *et al*, 2014).

Proteomic analyses of dentine have identified the presence of a wide array of various constituents with potential roles in orchestrating bone repair and mineralisation, including transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ), bone morphogenetic protein-2 (BMP-2), biglycan and decorin (Park *et al*, 2009; Casagrande *et al*, 2010; Jagŕ *et al*, 2012). This Chapter aimed to determine the bioactive nature of dentine matrix by assessing the effects observed when applied to fibronectin-adherent (FNA) BMMSCs (as characterised in Chapter 2). Effects of DDM on cellular expansion, migration and apoptosis were investigated. Crucially, the potency of DDM to stimulate differentiation on BMMSCs was analysed, with the concentration required for observed effects additionally elucidated. Determining the potential bioactive nature of dentine matrix on BMMSCs provided an indication to the potential for enhancing the bone repair process.

#### 3.2 Materials and Methods

#### 3.2.1.1 Ethical Approval and Tooth Acquisition

Human teeth were obtained from the Cardiff University, School of Dentistry Governance Committee, following ethical approval from the Research Ethics Committee, under South East Wales Tissue Bank approval 12/WA/0289. Teeth were stored at -80°C, prior to processing.

#### 3.2.1.2 Preparation of Demineralised Dentine Matrix (DDM)

All residual soft tissues were removed using a scalpel and teeth were washed thrice in 1% (w/v) sodium azide (Sigma-Aldrich, UK), prior to further processing. Teeth were embedded in wax and the upper crown sectioned off, followed by partitioning of the whole tooth down the saggital plane, using an IsoMet low speed saw with a diamond tipped blade (Buehler, USA). Exposed pulp tissue was removed using tweezers and tooth sections were washed once in 1% (w/v) sodium azide (Sigma-Aldrich). The resulting tooth sections were individually processed by mechanical removal of cementum and remaining enamel material using a dental hand-piece equipped with a slow-speed diamond burr. Isolated dentine sections were frozen in liquid nitrogen for 10 min and then powdered in a 6750 Freezer/Mill (Spex CertiPrep, USA), impacted at intensity 100 for 2 min. Resulting dentine powder from 10 teeth (approximately 7.5g) was split equally into two 50mL tubes (Sarstedt, UK). Dentine matrix components from each tube were extracted using 40mL of a 7.5% EDTA disodium salt (Fisher Scientific, UK) solution, supplemented with the protease inhibitors 1mM iodoacetic acid (Sigma-Aldrich), 5mM n-ethylmaleimide (Sigma-Aldrich) and 5mM benzamidine-hydrochloride (Sigma-Aldrich), at 4°C, with gentle agitation for a total of 14 days. On alternate days, the tubes were centrifuged at 1,100g for 10 min and supernatant collected and fresh extraction solution was applied. Pooled supernatants were exhaustively dialysed using 12,000Da molecular weight cut-off (MWCO) dialysis tubing (Sigma-Aldrich), against repeated changes of deionised distilled H<sub>2</sub>O (ddH<sub>2</sub>O) for 10 days at 4°C. Dialysed extracts were lyophilised and resulting DDM was stored at -20°C.

#### **3.2.1.3** DDM Reconstitution and Concentration Determination

DDM was reconstituted in tris buffered saline (TBS) to an anticipated concentration of approximately 1mg/mL, vortex mixed for 30 s and sterile filtered through a 0.22µM syringe filter (Millipore, USA). Protein concentration was determined using a Pierce<sup>®</sup> BCA Protein Assay Kit (Thermo Fisher Scientific, USA), following manufacturers protocol. Briefly, 25µL of a reconstituted protein sample was pipetted into a 96-well plate (Sarstedt) in triplicate. Pre-prepared bovine serum albumin (BSA) standards (25µg-2,000 µg/mL) represented a standard curve. 200µL of Working Reagent was added to each well; the plate was mixed on a plate shaker at 750rpm for 30 s and incubated at 37°C for 30 min. The plate was cooled to ambient temperature and colorimetric absorbance at 562nm read using a SPECTROstar Omega Plate Reader (BMG Labtech, Germany). Absorbance readings were blank corrected and averaged; and protein concentrations calculated from the generated standard curve equation with Microsoft<sup>®</sup> Excel<sup>®</sup> 2010.

#### 3.2.2 Cellular Expansion

BMMSCs (10-10.5 population doublings, PDs) were seeded in triplicate into 96-well plates, at 4x10<sup>3</sup> cells/cm<sup>2</sup> in 5% foetal bovine serum (FBS) (Invitrogen, UK) culture media, conditioned with DDM at 10µg/mL, 1µg/ml, 0.1µg/mL and 0µg/mL (control). Media changes were performed every 48 h. After 0, 24, 48, 72 and 96 h, culture medium was aspirated and replaced with fresh culture medium supplemented with 20µL of thiazolyl blue tetrazolium blue (MTT) assay reagent (Sigma-aldrich) (5mg/mL MTT in phosphate buffered saline (PBS)). Plates were incubated for 5 h under standard culture conditions. Culture medium was aspirated and replaced with 100µL of dimethyl sulfoxide DMSO (Fisher Scientific) and incubated for 30 min under standard culture conditions. Plates were cooled to ambient temperature and colorimetric absorbance at 540nm was read using a FluoSTAR Omega Plate Reader (BMG Labtech). Wells incubated with MTT containing culture media without cells served as a blank reading. The assay was performed on three separate occasions.

#### 3.2.3 Apoptotic Activity

A Caspase-Glo<sup>®</sup> 3/7 Assay (Promega, UK) was used to quantify apoptotic activity of cells cultured in varying concentrations of DDM-conditioned media. Caspase-Glo<sup>®</sup>

3/7 Reagent was prepared by thawing the Caspase-Glo<sup>®</sup> 3/7 Buffer and adding the entire contents to Caspase-Glo<sup>®</sup> 3/7 Substrate, with gentle agitation to ensure complete mixture of the reagents. Prepared Caspase-Glo<sup>®</sup> 3/7 Reagent was stored for up to 1 week at 4°C, with warming to ambient temperature before use. BMMSCs (14-15 PDs) were seeded in triplicate into white-walled, optically clear 96-well plates (Corning, USA), at 4x10<sup>3</sup> cells/cm<sup>2</sup> in 10% FBS culture media conditioned with DDM at concentrations of 10µg/mL, 1µg/mL, 0.1µg/mL and 0µg/mL (control). After 6, 24 and 48 h, plates were removed from incubation and cooled to ambient temperature. 50µL of Caspase-Glo<sup>®</sup> 3/7 Reagent was added to each well and the plate was incubated on a plate shaker at 300rpm for 30 s followed by incubation at room temperature for 1 h, protected from light. The plate was transferred to a FluoSTAR Optima Plate Reader (BMG Labtech) set to maximum optical gain settings, and arbitrary relative luminescence units (RLUs) were measured. Wells containing no cells incubated with Caspase-Glo<sup>®</sup> 3/7 reagent and culture media served as a blank reading. The assay was performed on three separate occasions.

#### 3.2.4 Cellular Migration

24-well tissue culture inserts (Boyden inserts) with 8µM pores (Greiner Bio One, Austria), were coated with 10µg of type I collagen from rat-tail (Sigma-Aldrich). Type I collagen was supplied as a 3.7mg/mL stock solution in 0.02N acetic acid. For 1.08mL of collagen gel, 52.54µL of type I collagen was added to 1,016.88µL of  $\alpha$ -MEM and then neutralised with 10.58µL of 0.1N sodium hydroxide (NaOH) solution (Fisher Scientific). 60µL of collagen solution was applied to the upper membrane of each cell culture insert, which were subsequently placed in 24-well plates (Sarstedt) and incubated at 37°C overnight to allow solidification of collagen. Excess culture media was aspirated and cell culture inserts were allowed to air dry, under laminar flow, for 1 h at room temperature. BMMSCs (12-13.5 PDs) were cultured with serum-free media for 24 h, passaged and seeded at 10<sup>4</sup> cells/cm<sup>2</sup> in 200µL of serum-free culture media in the upper compartment of collagen-coated inserts. 600µL of serum-free culture media conditioned with DDM at 10µg/mL, 1µg/mL, 0.1µg/mL and 0µg/mL (control), was added to the lower compartment and the culture plates were incubated under standard culture conditions for 20 h. Figure 3.1 depicts the layout of a Boyden insert.



Figure 3.1 - Schematic diagram demonstrating the layout of a Boyden insert placed into a tissue culture plate. Cells were seeded into the upper chamber and were able to migrate through the collagen coated permeable membrane of the insert into the lower chamber. Migrated cells were fluorescently labelled for quantification.

Culture media was aspirated from the lower compartment and replaced with 450µL of serum-free culture media supplemented with 8µM calcein-AM (Sigma-Aldrich); and incubated under standard culture conditions for 45 min. Culture media was removed from the lower compartment and replaced with 500µL of trypsin (Sigma-Aldrich) diluted 1:5 in PBS, per well. Plates were incubated for 10 min at 37°C, followed by gentle agitation and 200µL of trypsin/PBS solution from each well was removed and placed into a well of a black-walled optically clear 96-well plate (Corning). The plate was transferred to a FluoSTAR Optima plate reader (BMG LABTECH) and fluorescence read using 485nm excitation/520nm emission wavelengths. The experiment was performed on three separate occasions.

#### 3.2.5 Mineral Deposition

Basal media (non-osteogenic) was prepared by supplementing  $\alpha$ -MEM with 10% FBS and 1% antibiotic/antimycotic solution. Osteogenic media (positive control) was prepared by supplementing basal media with 10mM  $\beta$ -glycerophosphate (Sigma-

Aldrich) and 10nM dexamethasone (Sigma-Aldrich). DDM-conditioned media was prepared by supplementing basal media with DDM at final concentrations of 10  $\mu$ g/mL, 1  $\mu$ g/mL and 0.1  $\mu$ g/mL.

BMMSCs (15.2-16.5 PDs) were seeded into 24-well tissue culture plates and cultured in 10, 1 and 0.1  $\mu$ g/mL DDM, osteogenic media (positive control) and unsupplemented media (negative control) for 28 days. Media changes were performed every 2 days. After 28 days, media was aspirated and cells washed twice with PBS. Cells were fixed in 10% formaldehyde (Sigma-Aldrich) for 30 min at room temperature. Formaldehyde was aspirated and cells were washed twice with ddH<sub>2</sub>O and used immediately for Alizarin red staining.

#### 3.2.5.1 Alizarin Red Staining

500μL of 2% (w/v) Alizarin red S (pH 4.1-4.3) (Sigma-Aldrich) was added to each well and incubated for 20 min with gentle agitation. Alizarin red solution was aspirated and cells washed five times for 5 min each with ddH<sub>2</sub>O, or until wash solution was colourless. All residual wash solution was aspirated and the plate air-dried under ambient conditions. Cells were photographed under light microscopy, using a Panasonic DMC-G1 Camera.

#### 3.2.6 Differentiation Gene Expression

BMMSCs (16.5-17 PDs) were seeded at  $10^4$  cells/cm<sup>2</sup> into 6-well or 12-well culture plates (Sarstedt) and cultured in un-supplemented media (negative control), osteogenic induction media (positive control) and DDM supplemented media at a final concentration of 10 µg/mL. Media was changed every 2 days. After 2, 5 and 28 days, RNA was extracted and converted to cDNA via reverse transcription, as described previously (Sections 2.2.3 - 2.2.4). Resulting cDNA was diluted 1:10 with nuclease-free water (Promega) (1:5 dilution for 500ng of cDNA). For each quantitative real time-polymerase chain reaction (q-PCR), 2µL of 3µM primers (forward and reverse sequences, detailed in table 3.1) were mixed with 10µL of 2x PrecisionFAST q-PCR Master Mix (Primer Design, UK) and 1µL of nuclease-free water. 5µL of diluted cDNA was pipetted in triplicate into white 96-well q-PCR plates (Primer Design), followed by 15µL of pre-prepared Master Mix. Plates were covered with adhesive optical seals (Primer Design); and centrifuged at 500*g* for 3 min. q-PCR runs were performed in a QuantStudio<sup>™</sup> 6 Flex Real-Time PCR System (Thermo Fisher Scientific), using QuantStudio<sup>™</sup> Real-Time PCR Software (v1.0). Reaction conditions were as follows: one initial denaturation at 95°C for 20 s, forty cycles of denaturation for 1 s and annealing for 20 s. After cycling was completed, melt curve analysis was performed as follows: 95°C denaturation for 15 s, 60°C dissociation for 1 min and 95°C denaturation for 15 s. Primer sequences are detailed in Table 3.2.1. GAPDH was used as an internal reference for normalisation of data.

Gene	Primer sequence: 5'-3'	Annealing	Product	Primer
product		temperature	length	designer
		(°C)	(bp)	
САРОН		55	96	Author
GAPDIT		55	30	Aution
	R: GTGACCAGGCGCCCAATACGA			
RunX2	F: GGTTAATCTCCGCAGGTCACT	55	80	Author
	R: CCCTCTGTTGTAAATACTGCTTGC			
OPN	F: TCTCCTAGCCCCACAGAATG	55	80	Author
	R: CGTTGGACTTACTTGGAACGG			
COL1A1	F: TGCTGTTGGTGCTAAGGG	55	177	Dr. Wayne
				Ayre
				(Cardiff
				University)
OC	F: GGCAGCGAGGTAGTGAAGAG	55	102	Dr. Wayne
	B. CTCACACACCTCCCTCCT			Ayre
				(Cardiff
				University)

Table 3.1 – Table detailing primer sequences used for q-PCR analysis of genes expressed during osteogenic differentiation of BMMSCs.

#### 3.2.7 Statistical Analyses

Statistical analyses were performed utilising One-Way ANOVA, with a post-hoc Tukey test when comparing 3 or more groups, or a 2-tailed T-test when comparing 2 groups, using GraphPad InStat 3 (v3.06). Statistical values were defined as significant (\*, P<0.05), very significant (\*\*, P<0.01), or extremely significant (\*\*\*, P<0.001).

#### 3.3 Results

#### 3.3.1 Effect of DDM on Cellular Expansion

Over 96 h in culture, DDM evoked a reduction of BMMSC expansion in an observed dose-dependent manner. After 24 h, DDM at  $10\mu$ g/mL resulted in a significant reduction in cell expansion, compared to cells cultured with DDM at  $1\mu$ g/mL (P<0.001) and  $0.1\mu$ g/mL (P<0.01), and controls (P<0.001). After 48 h, expansion of BMMSCs cultured with DDM at  $1\mu$ g/mL was significantly reduced, compared to BMMSCs cultured in un-conditioned media (P<0.05). After 72 h, DDM at  $0.1\mu$ g/mL reduced BMMSC expansion compared to controls (P<0.05). After 96 h, DDM at  $1\mu$ g/mL reduced expansion compared to cells cultured with  $0.1\mu$ g/mL DDM; however, expansion of cells with DDM at  $0.1\mu$ g/mL was not significantly different compared to controls (P<0.05) (Figure 3.2).





#### 3.3.2 Apoptotic Activity of BMMSCs Cultured with DDM

DDM-conditioned media did not increase caspase 3/7 activity of BMMSCs after 6 and 24 h in culture. After 48 h in culture, BMMSCs cultured in media conditioned with 10µg/mL DDM demonstrated a statistically significant reduction of caspase-3/7 enzyme activity, compared to BMMSCs cultured with un-conditioned media (P<0.05) (Figure 3.3).



Figure 3.3 - Apoptotic activity of BMMSCs cultured with DDM. No increase in apoptotic activity was observed for BMMSCs cultured in DDM up to  $10\mu g/mL$ . Apoptotic activity was demonstrated to be significantly lower in BMMSCs cultured in DDM at  $10\mu g/mL$ , compared to cells cultured in un-conditioned media after 48 h. Error bars represent ±SD of mean (n=3). \*= P<0.05.

#### 3.3.3 Migratory Activity of DDM on BMMSCs

DDM provoked an inverse dose-dependent effect on migratory behaviour of BMMSCs; migration decreased with increasing DDM concentration. DDM at  $0.1\mu g/mL$  enhanced migratory behaviour of cells (P<0.05), whereas DDM at  $10\mu g/mL$  suppresses migratory activity (P<0.05), compared to un-conditioned controls. DDM at  $1\mu g/mL$  had no effect on migratory behaviour of BMMSCs (Figure 3.4).



Figure 3.4 - Migratory effect of DDM on BMMSCs. DDM at  $0.1\mu g/mL$  enhances migration of cells compared to controls, however, DDM at  $10\mu g/mL$  appears to be anti-migratory. DDM at  $1\mu g/mL$  had no effect on BMMSC migration. Error bars represent ±SD of mean (n=3). \*= P<0.05, \*\*\* = P<0.001.

#### 3.3.4 Osteogenic Potency of DDM towards BMMSCs

After 28 days, BMMSCs cultured in osteogenic differentiation media stained with Alizarin red demonstrated brightly red stained nodules of mineralisation, indicative of successful osteogenic differentiation. BMMSCs cultured in 10µg/mL DDM showed minor mineral deposition, as indicated by faint Alizarin red staining of distinctly localised foci. No Alizarin red staining was evident for BMMSCs cultured in DDM at 1µg/mL, 0.1µg/mL or basal media (Figure 3.5). Thus, 10µg/mL DDM was chosen to assess changes in gene expression during the osteogenic differentiation process.

After 2 days in culture, gene expression of RunX2 was not enhanced in BMMSCs cultured with either osteogenic differentiation media or media conditioned with 10µg/mL DDM (Figure 3.6). Expression of RunX2 was up-regulated by osteogenic differentiation media (P<0.001) and 10 µg/mL DDM (P<0.05), relative to basal media after 5 days in culture.

After 28 days in culture, BMMSCs cultured in 10µg/mL DDM demonstrated a statistically significant up-regulation of collagen type I (COL1A1) expression relative to cells in basal media (P<0.05). An increase of osteocalcin (OC) expression is also observed for BMMSCs cultured in DDM, with up-regulation approaching statistical significance (P=0.06). Conversely, osteopontin (OPN) expression was up regulated in BMMSCs cultured in un-conditioned media relative, to BMMSCs cultured 10µg/mL DDM culture (P<0.05) (Figure 3.7). Gene expression analysis of BMMSCs cultured in osteogenic differentiation media for 28 days was not performed due to difficulties for the extraction of RNA from the heavily mineralised matrix.







Figure 3.6 - Expression of RunX2 in BMMSCs cultured in DDM-conditioned, osteogenic, and basal media for 2 and 5 days. After 2 days in culture, RunX2 gene expression was not changed in BMMSCs cultured in DDM supplemented or osteogenic induction media, compared with BMMSCs cultured in un-supplemented media. RunX2 expression is significantly increased in BMMSCs cultured in both DDM-conditioned media and osteogenic induction media, compared with BMMSCs cultured in both DDM-conditioned media. Error bars represent  $\pm$ SD of mean (n=3). \*= P<0.05, \*\* = P<0.01, \*\*\* = P<0.001.



Figure 3.7 - Expression of OPN, COL1A1 and OC mRNA in BMMSCs cultured in  $10\mu g/mL$  DDM media and basal media after 28 days. OPN expression for cells cultured with DDM is down-regulated, compared to controls (P <0.05), however COL1A1 is increased (P < 0.05). OC up-regulation approaches significance in DDM-treated cells (P=0.06). Error bars represent ±SD of mean (n=4). \*= P<0.05.

#### 3.4 Discussion

Although several *in vivo* studies have identified the osteogenic potency of dentine matrix for augmentation of bone repair, little is currently known regarding the influences of DDM on BMMSCs. Repair of bone *in vivo* is a complex and highly organised process involving the migration, proliferation and differentiation of BMMSCs. Within this Chapter, DDM has been demonstrated to induce migration, reduce cell expansion and attenuate apoptosis of BMMSCs. Critically, the highest tested concentration of 10µg/mL demonstrated osteogenic responses and mineralised matrix deposition by BMMSCs.

In this study, it was demonstrated that DDM induced migration of BMMSCs in an inverse dose-dependent manner with DDM at 0.1µg/mL stimulating migration of BMMSCs. However, 10µg/mL DDM elicited anti-migratory effects on BMMSCs. MSCs are able to migrate towards sites of bone fracture, where they differentiate and participate in repair of the bone fracture site (Kumagai et al, 2008; Granero-Moltó et al, 2009; Alm et al, 2010). Although the mechanisms of migration are not fully understood, MSC homing is acknowledged to occur through chemokine signalling (Honczarenko et al, 2006; Ponte et al, 2007) and hypoxia (Rochefort et al, 2006). The matrix of dentine is additionally acknowledged as a reservoir of proinflammatory cytokines, including the interleukins (IL-) IL-1 $\alpha$ , IL-1 $\beta$ , IL-4, IL-6 and IL-8 (Graham et al, 2007). Odontoblasts are reported to constitutively express IL-8, which can be up-regulated following exposure to *E. coli*-derived lipopolysaccharide (LPS) and it postulated be become sequestered within dentine (Levin *et al*, 1999). Migration of human BMMSCs in vitro is inducible by IL-6 (Rattigan et al, 2010) and IL-8 (Anton et al, 2012); and therefore, resident cytokines within DDM may be acting as chemotactic agents of BMMSCs. Growth factors, such as TGF- $\beta_1$ , have been demonstrated to induce migration of dental papilla-derived cells (Kwon et al, 2010) and dental pulp-derived, stem cells (DPSCs) (Howard *et al*, 2010). TGF- $\beta_1$  is additionally a pertinent component of bone matrix, which can induce migration of osteoblast-like cells (Pfeilschifter et al, 1990) and BMMSCs; and is postulated to couple bone resorption with formation to maintain the balance of bone remodeling (Tang et al, 2009). Fibroblast growth factor (FGF) is reported to be present within

both bone and dentine matrices (Roberts-Clark and Smith, 2000; Shimizu-Sasaki *et al*, 2003) and has been implicated in stimulating migratory behaviour of dental pulp-derived cells (Howard *et al*, 2010; Shimabukuro *et al*, 2009). Activation of FGF-receptor (FGFR) in dental pulp cells has been shown to increase formation of focal adhesions and enhance migration (Zhang *et al*, 2015a), potentially indicating a pertinent role for FGF in providing chemotactic stimuli. Furthermore, migratory behaviour of pulpal cells is noted to be promoted by dentine sialoprotein (DSP) (Lee *et al*, 2012a) and dentine phosphophoryn (Yasuda *et al*, 2008); and may additionally be responsible for directing migration of BMMSCs. Although components of DDM are acknowledged to participate in the promotion of cellular migration, DDM at 10µg/mL demonstrated the opposite effect. This observation may be as a result of cells from a distant location towards the site of implantation. Additionally, higher concentrations may be inducing differentiation of BMMSCs, as opposed to stimulating migration.

The application of DDM to BMMSCs demonstrated a dose-dependent effect on the reduction of cell expansion over a 4-day incubation period. A decrease in expansion was observed for cells cultured with  $1\mu g/mL$  DDM after 48 h, which was maintained up to 96 h, whereas expansion was reduced for cells cultured with DDM at 0.1µg/mL after 72 h only, compared to controls. However, cell expansion was reduced for BMMSCs cultured with 10µg/mL DDM after 24 h in culture, with a marked reduction of expansion observed after 96 h. DDM at comparable concentrations has been shown to dose-dependently enhance expansion of DPSCs, with 10µg/mL providing the greatest effect (Lee et al, 2015). Other studies have acknowledged DDM as eliciting an inverse dose-dependent effect on cell expansion, identifying lower concentrations as stimulatory and higher concentrations being inhibitory for expansion of odontoblast-like cells, pulp-derived cells (Musson et al, 2010) and endothelial cells (Zhang et al, 2011a). The effects of expansion DDM on BMMSCs have been less well studied, although Yu et al, (2014) demonstrated that DDM up to 10µg/mL does not affect expansion of BMMSCs, although a reduction is noted for cells cultured with DDM at 100µg/mL. However, cells used by Yu et al,

(2014), represented an un-purified population and thus, results may differ from those demonstrated in this Thesis due to the immature nature of the BMMSCs. Demineralised tooth matrix (DTM) has been reported to decrease expansion of clonally-derived, BMMSCs, in a dose-dependent manner (Harrington, 2014). Even though no concentration of DDM tested in this study enhanced cell expansion, the reduction of expansion occurring with DDM in an apparent dose-dependent manner, predominantly at  $10\mu g/mL$ , suggests that this concentration may be suppressing expansion and stimulating alternative cell responses.

The observed effects of DDM attenuating BMMSC expansion may be attributable to an inverse relationship of an increase in cell apoptosis or initiation of differentiation, whereby differentiation down-regulates proliferation (Stein and Lian, 1993). Assessment of the activity of caspase-3/-7 in DDM-treated BMMSCs was performed to dismiss the observed reduction in expansion resulting from apoptotic activity. Over-conditioning of dentine with EDTA in vitro can decrease odontoblast survival (Murray et al, 2000), potentially as a result of over-stimulation from TGF- $\beta_1$  (He *et al*, 2005). TGF- $\beta_1$  can also induce apoptosis of BMMSCs by stimulating mitochondrial reactive oxygen species (ROS) production (Zhang et al, 2015b). In addition, apoptosis of osteoblasts occurs at sites of bone fracture and is postulated to act as an event coupled with osteoblast proliferation as a regulatory mechanism of callus formation and remodelling (Landry et al, 1997; Li et al, 2002), with BMP-2 identified as an inducer of osteoblast apoptosis (Haÿ et al, 2001). Attenuation of pro-apoptotic signals at bone regeneration sites can prolong matrix secretion and ossification, and thus prolong and enhance bone repair (Komatsu et al, 2007).

Culture of BMMSCs with DDM demonstrated a decrease in apoptotic activity compared to un-treated cells, with a dose-dependent effect observed. DDM at 5-10µg/mL has been reported to reduce apoptosis of DPSCs, in addition to reducing expression of caspase-3 and increasing expression of Akt-1 (Lee *et al*, 2015). Activation of Akt-1 is reported as one of the principal pathways for encouraging cell survival and tolerance of apoptotic stimuli (Song *et al*, 2005). Platelet-derived growth factor (PDGF) has been implicated in activation of Akt-1 and Akt-2 signalling

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pathways (Romashkova and Makarov, 1999). Recombinant human DSP can activate signalling to result in the phosphorylation and activation of Akt-1 in human DPSCs (Lee *et al*, 2012a); and additionally in human cementoblasts, when combined with BMP-2 (Lee *et al*, 2014). High inorganic phosphate, which results from carious demineralisation of teeth (Larmas, 2001), reduces viability and increases apoptosis of MO6-G3 odontoblast-like cells (Bourgine *et al*, 2011). However, dentine matrix protein-1 (DMP-1) attenuates phosphate-induced apoptosis of odontoblasts (Rangiani *et al*, 2012). Dentine phosphoprotein (DPP) has suppressive activities against phosphate-induced apoptosis of odontoblast-like cells (Fujisawa *et al*, 2009), in addition to activating Akt in pre-odontoblasts (Eapen and George, 2015).

When cultured in the presence of 10µg/mL DDM for 28 days, BMMSCs elicited a detectable mineralised matrix deposition as indicated by Alizarin red staining. DPSCs cultured in the presence of DDM are noted to deposit mineralised matrix (Lee *et al*, 2015), concomitant with the ability of dentine to induce reparative matrix production (Smith et al, 1995; Goldberg et al, 2011). The potency of DDM to provoke mineral deposition of DPSCs was not explored in this Thesis. However, EDTA soluble proteins from whole tooth preparations have been reported to elicit less potent in vitro differentiation of MSCs derived from bone marrow and adipose tissue, compared to MSCs derived from the dental pulp (Chun et al, 2009). DPSCs produce a dense mineralised matrix when cultured in media conditioned with DTM, in addition to significantly increased gene expression of OC. Though, clonal BMMSCs only develop small and sparse mineralised nodules when cultured with comparable concentrations of DTM (Harrington, 2014). The greater potency of DTM to induce mineralisation of DPSCs can be potentially attributable to the hierarchical model of differentiation potential, whereby a small proportion of heterogeneous DPSCs is postulated to consist of highly proliferative, multipotent cells present within a larger proportion of progenitors with more restricted lineage commitment (Gronthos et al, 2002). Within this Thesis, the osteogenic effects of DDM were performed on primitive BMMSCs and thus, the effects of differentiation may be less pronounced.

10µg/mL DDM was shown to up-regulate gene expression of the early osteogenic marker, RunX2, in BMMSCs after 5 days in culture, compared to un-treated cells. As a marker of early osteogenic commitment, the ability to up-regulate RunX2 expression indicates potential for DDM to direct BMMSC differentiation. Odontoblastic differentiation and subsequent mineralised nodule production of dental pulp-derived cells is additionally associated with an increase in RunX2 expression (Zhang et al, 2007b; Han et al, 2014). RunX2 expression is associated with exit from the cell cycle and osteoblast maturation (Pratap et al, 2003); and thus, may be responsible for the decline in expansion observed for BMMSCs cultured in the presence of DDM. After 28 days in culture, 10µg/mL DDM increased mRNA expression of COL1A1 accompanied with a statistically non-significant increase in OC expression, compared to cells cultured in control media. The slightly elevated expression of OC in BMMSCs cultured with DDM correlates with the low abundance of mineralised matrix detected by Alizarin red staining and hence, demonstrates a mild osteoinductive effect in vitro. OPN mRNA expression was down regulated in DDM-treated cells, compared to controls. OPN expression is acknowledged to peak during early osteogenic differentiation, followed by a decrease in expression during later differentiation (Nakamura et al, 2009). Therefore, this suggests that BMMSCs in control culture were beginning to increase commitment towards the osteoblast lineage as an artefact of increased expansion and progression down the lineage hierarchy (as discussed in Chapter 2). Furthermore, OPN is reported to inhibit ectopic calcification *in vivo* and can actively block the growth of hydroxyapatite crystals (Steitz et al, 2002). Therefore, down regulation of OPN in DDM-treated BMMSCs suggest permission of mineralisation. Taken together, the up-regulation of osteogenic markers RunX2, COL1A1 and deposition of a mineralised matrix indicates the bioactive nature of DDM for directing differentiation of BMMSCs towards the osteoblast lineage, which drives the bone repair process.

This Chapter has demonstrated that supplementation of DDM *in vitro* can alter cellular behaviour of BMMSCs that favours osteogenesis. DDM reduces expansion of BMMSCs, whilst not inducing an apoptotic response. However, DDM at 10µg/mL

induced differentiation characterised by up-regulation of osteogenic genes and deposition of mineralised matrix. DDM was additionally migratory at low concentrations. These observed cellular effects largely correlate with the events that occur during bone repair, by which undifferentiated can migrate to the site of injury and differentiate into cells that can participate in tissue repair by producing a mineralised ECM. Collectively, the responses of BMMSCs to DDM indicate high potential for augmentation of bone repair. Numerous non-collagenous proteins (NCPs) are secreted into dentine, such as DSP/DPP (MacDougall *et al*, 1997), DMP-1 (George *et al*, 1993); and a plethora of growth factors, including TGF- $\beta_1$ , FGF-2, PDGF and VEGF (Finkelman *et al*, 1990; Roberts-Clark and Smith, 2000). Subsequent Chapters in this Thesis have investigated some of these proposed bioactive components of DDM and elucidate their role in directing the process of bone repair.

## Chapter 4 - Determination of Bioactive Constituents in Demineralsed Dentine Matrix (DDM) for Directing Biological Activity of Bone Marrow-Derived, Mesenchymal Stem Cells (BMMSCs)

#### 4.1 Introduction

The process of mineralised tissue formation and repair constitutes a complex cascade of events that ultimately lead to the functional production and regeneration of tissue, respectively. Amongst other elements, growth factors are fundamental mediators in coordinating development and regeneration of bone and teeth. Although not exhaustive, the growth factors transforming growth factor-  $\beta_1$  (TGF- $\beta_1$ ) (Bostrom and Asnis, 1998), bone morphogenetic proteins (BMPs) BMP-2, BMP-4, BMP-7 (Lian *et al*, 2006), fibroblast growth factor (FGF) (Naganawa *et al*, 2006) and vascular epidermal growth factor (VEGF) (Gerber *et al*, 1999), are instrumental in the regulation of bone. Moreover, TGF- $\beta_1$  (Tziafas *et al*, 1998), BMP-2 (Casagrande *et al*, 2010) and BMP-7 (Six *et al*, 2002), are implicated in possessing roles for mediating reparative dentine formation.

Although growth factors present within bone and dentine matrix are acknowledged as potent cell signalling molecules, additional proteins present in both matrices are proposed to participate in modulation of growth factor activity. Of note, biglycan and decorin are reported to bind to TGF- $\beta_1$  (Hildebrand *et al*, 1994; Baker *et al*, 2009), in addition to type I collagen (Schönherr *et al*, 1995; Svensson *et al*, 1995). Biglycan and decorin are hypothesised to sequester growth factors within the matrix and therefore, modulate their availability (Hildebrand *et al*, 1994; Baker *et al*, 2009).

Heparin is a highly sulphated glycosaminoglycan (GAG) with alternatively modified sulphation patterns which can recognise specific structures (Ashikari-Hada *et al*, 2004). Sulphated heparin and is able to bind a wide variety of growth factors, including TGF- $\beta_1$  (McCaffrey *et al*, 1992), BMP-2 (Ruppert *et al*, 1996), FGF-2 and platelet-derived growth factor (PDGF) (Rajesh *et al*, 1995). The mechanism for growth factor binding is proposed to be a result of electrostatic interactions

between negatively charged sulphate groups and positively charged amino acids on proteins (Jeon *et al*, 2011). Heparin-affinity has successfully been exploited to isolate growth factors from bone matrix, with the isolated factors demonstrating bioactivity with regards to proliferation of endothelial cells and mitogenic potential for osteoblasts (Hauschka *et al*, 1986).

Even though numerous growth factors have been identified in dentine matrix, no study has determined the concentrations of a range of osteogenic inducing factors and thus, the ratios of specific factors within the matrix that are postulated to contribute towards mineralised tissue repair is yet to be elucidated. Methods used in this Chapter quantified the abundance of a panel of potent osteogenic growth factors within a demineralised dentine matrix (DDM) preparation, using enzyme-linked immunosorbent assays (ELISAs), thus revealing the optimal physiological ratios of these factors anticipated to orchestrate the induction of mineralised tissue formation. To determine whether these growth factors possess potency when isolated from dentine, growth factors were depleted from DDM via heparin-affinity chromatography and responses of bone marrow-derived, mesenchymal stem cells (BMMSCs) to fractionated DDM reassessed and compared to un-fractionated DDM. Critically, experimental procedures within this Chapter aimed to clarify a requirement for additional factors in regulating cellular responses to growth factors.

#### 4.2 Materials and Methods

#### 4.2.1 DDM Sample Preparation

DDM was obtained from human teeth, as previously described (Section 3.2.1.2). DDM was reconstituted in deionised distilled H<sub>2</sub>O (ddH<sub>2</sub>O) to an anticipated concentration of approximately 1mg/mL, mixed by vortex for 30 s and sterilefiltered through a 0.22µm syringe filter (Millipore, USA). DDM solution was assayed for protein concentration, using a Pierce<sup>®</sup> BCA Protein Assay Kit (Thermo Fisher Scientific, USA) as previously described (Section 3.2.1.3). Aliquots of known concentration of DDM solution were pipetted into sterilised 1.5mL tubes (Eppendorf, UK), frozen at -80°C and lyophilised.

## 4.2.2 Protein Separation with Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2x Laemmli Sample Buffer (65.8mM tris-hydrochloride (Sigma-Aldrich, UK); pH 6.8, 2.1% sodium dodecyl sulphate (SDS) (Fisher Scientific, UK), 26.3% (w/v) glycerol (Fisher Scientific), 0.01% bromophenol blue (Sigma-Aldrich)) (BioRad Laboratories, USA) was supplemented with 2-mercaptoethanol (Sigma-Aldrich) at 5% (v/v) and diluted 1:1 with ddH<sub>2</sub>O to generate 1x Laemelli Sample Buffer. DDM was reconstituted in 1x Laemmli Sample Buffer at a concentration of 0.67µg/µL (for silver staining, Section 4.2.2.1) or 2µg/µL (for protein electroblotting, Section 4.2.2.2). DDM/Laemmli Buffer was heated to 95°C for 5 min and 15µL loaded into 4-15% Mini Protean TGX pre-cast gels (BioRad Laboratories), alongside 7.5µL of Kaleidoscope Precision Plus Pre-Stained Protein Standards (BioRad Laboratories). Gels were placed into Mini-PROTEAN<sup>®</sup> Tetra Vertical Electrophoresis Cell (BioRad Laboratories) in 10x SDS-PAGE running buffer (250mM tris-base (Sigma-Aldrich), 1.92M glycine (Fisher Scientific), 1% SDS, pH 8.3), diluted to a 1x working concentration in ddH<sub>2</sub>O. Proteins were separated by electrophoresis at 200V for 30-35 min, until the migration front reached the bottom of the gel.

#### 4.2.2.1 Silver Staining

Silver staining was performed using a Silver Stain Plus<sup>™</sup> kit (BioRad Laboratories), following manufacturer protocol. The SDS-PAGE gel was removed from casts and

placed in fixative solution (50% v/v methanol (Fisher Scientific), 10% v/v acetic acid (Fisher Scientific), 1% v/v Fixative Enhancer Concentrate, 30% v/v ddH<sub>2</sub>O) with gentle agitation for 20 min. The fixed gel was washed twice with 400mL ddH<sub>2</sub>O for 10 min per wash. Staining solution was prepared by adding 5mL Silver Complex Solution, 5mL Reduction Moderator Solution and 5mL Image Development Reagent to 35mL ddH<sub>2</sub>O, with gentle stirring followed by addition of 50mL Development Accelerator Solution to Staining solution. The gel was placed in staining solution with gentle agitation for around 20 min until desired staining intensity was achieved. Staining reaction was stopped by placing the gel in 5% v/v acetic acid for 15 min with a final wash in ddH<sub>2</sub>O for 5 min. The gel was imaged with a Gel Doc EZ System (BioRad Laboratories).

#### 4.2.2.2 Protein Electrobloting

Gels used for protein electroblotting were removed from casts and soaked in transfer buffer (25mM tris-base, 192mM glycine, 20% (v/v) methanol, pH 8.3) for 10 min. Two electroblot sponges, two sheets of thick blot filter paper (BioRad Laboratories) and nitrocellulose membrane (GE Healthcare, UK), were additionally soaked in transfer buffer for 5 min. One electroblot sponge was placed onto the back plate of a blotting module, followed by one sheet of filter paper, the TGX gel containing the proteins separated by electrophoresis, nitrocellulose membrane and a sheet of filter paper and finally the remaining electroblot sponge, thereby preparing an electroblot 'sandwich'. The blotting module was clamped shut and placed into the Mini-PROTEAN® Tetra Vertical Electrophoresis Cell and transferred at 100V constant for 1 h. Protein transfer was confirmed by staining of the nitrocellulose with Ponceau Red S stain for 5 min, followed by de-staining in tris buffered saline (TBS) by repeated washing.

#### 4.2.2.3 Immunoblotting for Protein Detection

Membranes were cut to size and placed into 50mL tubes (Sarstedt, UK). Nonspecific binding sites were blocked in 5% w/v non-fat dry milk (Tesco, UK), in TBS supplemented with 0.05% Tween-20 (Sigma-Aldrich) (TBS-T) solution for 1 h at room temperature, with gentle agitation. Membranes were transferred to blocking buffer solution with primary antibodies, as outlined in Table 4.1; and incubated overnight at 4°C, with gentle agitation. Membranes were washed four times for 5 min each in TBS-T, transferred to blocking buffer with respective secondary antibodies and incubated for 1 h at room temperature, as outlined in Table 4.1. Membranes were washed four times for 5 min each in TBS-T, with two additional 5 min washes in TBS. Membranes were treated ECL Reagent (GE Healthcare) for 3 min, sandwiched in development pouches and exposed to X-ray development film (GE Healthcare).

Negative controls were performed by incubating primary antibodies with 10X excess of respective blocking peptide for 1 h at room temperature, prior to protein detection. Blocking peptides are listed in Table 4.1.

To determine the molecular weight of proteins detected by silver staining/immunoblotting, the relative migration of the protein/band of interest was measured and compared to the calculated standard curve for the (Log) relative migration of molecular weight standards.

Primary	Primary Ab	Secondary Ab	Secondary Ab	Blocking
antibody (Ab)	Dilution		dilution	peptide
TGF-β <sub>1</sub> (V) -	1:200	Anti-rabbit	1:25,000	TGF-β <sub>1</sub> (V) -
Santa Cruz		HRP		Blocking
(USA)				Peptide
				Santa Cruz
BMP-2 (N-14)	1:500	Anti-goat HRP	1:50,000	BMP-2 (N-14) -
- Santa Cruz				Blocking
				Peptide
				Santa Cruz
VEFG (A-20) -	1:200	Anti-rabbit	1:25,000	VEFG (A-20) –
Santa Cruz		HRP		Blocking
				Peptide
				Santa Cruz

Table 4.1 – Detailing antibodies and respective dilutions/blocking peptides used for protein electroblotting of DDM.

#### 4.2.3 ELISA Quantification of Growth Factors in DDM

#### 4.2.3.1 TGF-β<sub>1</sub>

Determination of TGF- $\beta_1$  concentration in DDM was performed using a Human TGF- $\beta_1$  Platinum ELISA kit (eBioscience, UK), according to manufacturer's instructions. Standards, blanks and samples were assayed in duplicate. Incubation steps were performed at room temperature on a plate shaker at 400rpm, unless otherwise stated. 20µL of DDM samples were pipetted into 1.5mL tubes (Eppendorf) and mixed with 180µL of 1x Assay Buffer and 20µL of 1N hydrochloric acid (HCl) followed by 1 h incubation without shaking. Samples were neutralised with 20µL of 1N sodium hydroxide (NaOH). TGF- $\beta_1$  Standard supplied within the Kit was reconstituted in ddH<sub>2</sub>O to prepare a 4,000pg/mL stock, with working standards ranging from 2,000pg/mL to 31pg/mL prepared by serial dilution in 1x Assay Buffer. 1x Assay Buffer served as a blank. 60µL of 1x Sample Buffer was added to sample

wells, followed by 40µL of pre-treated sample of each sample group in duplicate. Micro-wells were sealed with adhesive film and incubated for 2 h. Samples and standards were aspirated, micro-wells washed four times with 400µL of 1x Wash Buffer, followed by addition of 100 µL of pre-prepared 1x Biotin Conjugate to each well. Micro-wells were sealed and incubated for 2 h. Biotin Conjugate was aspirated and micro-wells washed, as previous. 100µL of pre-prepared 1x Streptavidin-HRP was added to each micro-well and incubated for 1 h. Streptavidin-HRP was aspirated and micro-wells washed as previous, followed by addition of 100µL of TMB Substrate Solution to each well. Micro-wells were incubated in the dark for 30 min without shaking, followed by addition of 100µL of Stop Solution. Absorbance at 450nm was read using a FluoSTAR OMEGA Plate Reader (BMG Labtech, Germany). Assay was performed on two separate occasions.

#### 4.2.3.2 Insulin-Like Growth Factor-1 (IGF-1)

Determination of Insulin-like Growth Factor-1 (IGF-1) concentration in DDM was performed using a Human IGF-1 Quantikine<sup>®</sup> ELISA Kit (R&D Systems, UK) according to manufacturer's instructions. IGF-1 Standard was reconstituted in 1ml of ddH<sub>2</sub>0 to create a 60ng/mL stock. 360µL of Calibrator Diluent RD5-22 was pipetted into a 1.5mL tube (Eppendorf). 200µL of Calibrator Diluent was added to six additional 1.5mL tubes (Eppendorf). 40µL of 60ng/mL stock IGF-1 was added to the tube containing 360µL of Calibrator Diluent RD5-22 and thoroughly mixed to generate a 6ng/mL standard. Additional standards were prepared by subsequent 1:1 dilutions with Calibrator Diluent RD5-22 to a concentration of 0.094ng/mL IGF-1. Calibrator Diluent RD5-22 served as a blank. 150µL of Assay Diluent RD1-53 was pipetted into all micro-wells. 50µL of each standard and sample was added to wells in triplicate and thoroughly mixed. Micro-well strips were sealed with adhesive film and incubated for 2 h at 4°C. Micro-wells were aspirated and washed four times with 400µL of 1x Wash Buffer, with gentle tapping against clean paper towel after the last wash step. 200µL of IGF-1 Conjugate (at 4°C) was added to each well, microwell strips were sealed with adhesive film and incubated for 1 h at 4°C. Micro-wells were aspirated and washed, as previous. 200µL of Substrate Solution was added to each well and incubated at room temperature in the dark for 30 min. 50µL of Stop

Solution was added to each well and absorbance at 450nm for primary wavelength (540nm as reference wavelength), was read using a FluoSTAR OMEGA Plate Reader (BMG Labtech). Assay was performed on two separate occasions.

#### 4.2.3.3 BMP-4 and BMP-7

Determination of BMP-4 and BMP-7 in human DDM was performed using Human BMP-4 and Human BMP-7 ELISA Kits, respectively (Sigma-Aldrich), following manufacturer's instructions. Incubations were performed at room temperature on a plate shaker at 100rpm unless otherwise stated. The following steps were carried out individually for each Kit. 5x Assay Diluent B was diluted 5-fold with ddH<sub>2</sub>O to prepare 1x Assay Diluent B. BMP-4/BMP-7 Standards were reconstituted in 400µL of 1x Assay Diluent B, to prepare a 50ng/mL stock standard. 80µL of 50ng/mL standard was thoroughly mixed with 586.7µL of Assay Diluent B, to prepare a 6,000ng/mL standard. Subsequent standards down to 8.23pg/mL were prepared by serial dilution of 6,000ng/mL in 1x Assay Diluent B. 1x Assay Diluent B served as blank. 100µL of each standard and sample was added to micro-wells in triplicate, sealed with adhesive film and incubated for 2.5 h. During incubation, Biotinylated Detection Antibody was reconstituted in 100µL of Assay Diluent B and diluted 65fold (BMP-4) or 80-fold (BMP-7), respectively, with 1x Assay Diluent B. Micro-wells were aspirated and washed four times with 300µL of 1x Wash Buffer, with gentle tapping of micro-wells onto clean paper towel after the final wash. 100µL of Biotinylated Detection Antibody was added to each well, which were sealed and incubated for 1 h. Wells were aspirated and washed, as previous. HRP-Streptavidin was diluted 80-fold (BMP-4) and 400-fold (BMP-7), respectively, with 1x Assay Diluent B; with 100µL being added to each well and incubated for 30 min. Microwells were aspirated and washed, as previous; and 100µL of ELISA Colorimetric TMB Reagent added to each well and incubated in the dark for 30 min. 50µL of Stop Solution was added to each well and absorbance at 450nm was read using a FluoSTAR OMEGA Plate Reader (BMG Labtech). Assays were performed on two separate occasions.

#### 4.2.3.4 BMP-2, VEGF, PDGF-BB and FGF-2

Determination of BMP-2, VEGF, PDGF-BB and FGF-2 in human DDM was performed using respective Human Mini ELISA Kits (PeproTech, USA), following manufacturer's instructions. Buffers used were obtained from an ELISA Buffer Kit (PeproTech). The following steps were carried out individually for each Kit. Incubations were performed at room temperature. Capture Antibody was reconstituted in ddH<sub>2</sub>O to a concentration of 100µg/mL. Capture Antibody was diluted to a working concentration of 0.5µg/mL in 1x Phosphate Buffered Saline (PBS), 100µL added to each well of a 96-well plate (PeproTech), sealed and incubated overnight. Wells were aspirated and washed four times with  $300\mu$ L of 1x Wash Buffer, followed by gentle tapping against a clean paper towel. 1x Block Buffer was sterile filtered using a syringe and 0.22µM pore filter (Millipore), prior to addition of 300µL per well. The plate was sealed with adhesive film and incubated for 1 h. During incubation, Recombinant Standard was reconstituted in ddH<sub>2</sub>O to a stock concentration of 1µg/mL. Recombinant Standards were diluted to concentrations of 4,000pg/mL (FGF-2), 2,000pg/mL (BMP-2) and 1,000pg/mL (VEGF and PDGF-BB) in 1x Diluent. 200µL of diluted stock standards were added to wells in triplicate. Wells were aspirated and washed as previous. 100µL of each standard, in triplicate, was serially diluted with 1:1 with 100µL 1x Diluent down to concentrations of 62.5ng/mL (FGF-2), 31.25ng/mL (BMP-2) and 15.63ng/mL (VEGF and PDGF-BB). 1x Diluent served as a blank. 100µL of each standard/sample was added to wells in triplicate; the plate was sealed with adhesive film and incubated for 2 h. Wells were aspirated and washed as previous. Detection antibody was diluted to a working concentration of 1µg/mL (BMP-2) or 0.25µg/mL (VEGF, FGF-2 and PDGF-BB) in 1x Diluent. 100µL of respective detection antibody was added to each well, the plate sealed and incubated for 2 h. Welld were aspirated and washed as previous. Avidin-HRP Conjugate was diluted 1:2,000 with 1x Diluent, 100µL was added to each well and plates were incubated for 30 min. Wells were aspirated and washed as previous, followed by addition of 100µL of ABTS Liquid Substrate to each well. Plates were incubated at room temperature for 25 min (FGF-2), 35 min (BMP-2 and VEGF) or 50 min (PDGF-BB); and absorbance determined at 405nm for primary wavelength

(650nm for reference wavelength) using a FluoSTAR OMEGA Plate Reader (BMG Labtech). Assays were performed on two separate occasions.

#### 4.2.3.5 Determination of Growth Factor Concentrations from ELISAs

For each assay, absorbance (for IGF-1, BMP-2, VEGF, PDGF-BB and FGF-2 assays, net absorbance readings were obtained by subtracting the reference wavelength from the primary wavelength) was plotted against standard concentrations. A standard curve to the polynomial of 3 was plotted between the points to generate a line of best fit with Microsoft<sup>®</sup> Excel<sup>®</sup> 2010. A R<sup>2</sup> value of >0.95 indicated sufficient correlation of the standard dilutions against absorbance. Absorbance values from the samples within the standard curve range were plotted against respective standard curves to obtain concentration values of growth actors within DDM. All values were scaled up to total abundance of growth factor per mg of DDM.

# 4.2.4 Fractionation of Heparin-Binding DDM Components via Affinity

#### Chromatography

Growth factors in DDM were fractionated by affinity chromatography using a Heparin HiTrap column (GE Healthcare), using a protocol adapted from Hauschka et al (1986). DDM was reconstituted in 30mL of binding buffer (0.01M trishydrochloride/0.1M sodium chloride (NaCl) (pH 7) in 18.2M $\Omega$  water); and sterile filtered through a 0.22µm pore syringe filter (Millipore). Protein concentration was determined using a Pierce<sup>®</sup> BCA Protein Assay Kit (Thermo Fisher Scientific), as described previously (Section 3.2.1.3). Heparin-affinity chromatography was performed, using AKTA Purifier apparatus controlled by Unicorn Software (GE Healthcare, v5.2). The system was washed in 20% (v/v) ethanol and pumped with 18.2M $\Omega$  water during connection of the Heparin HiTrap column. AKTA apparatus was equilibrated in binding buffer to determine baseline of conductivity and 280nm absorbance. DDM reconstituted in binding buffer at approximately 120µg/mL in 15mL was applied through the HiTrap column at a rate of 1mL/min, with flow through (FT) collected in 5mL fractions. DDM components bound to the column were eluted by a linear gradient increase of increasing NaCl concentration from 0.1-3M over twelve column volumes, at a rate of 2mL/min. Elution volumes were collected in 2.5mL fractions and subsequently pooled into two elution pools (EL1 and EL2).

Flow through and elution pools were transferred to 12,000Da molecular weight cutoff (MWCO) dialysis tubing (Sigma-Aldrich) and dialysed against ddH<sub>2</sub>0 for 2 days at 4°C, with continuous agitation and dialysis solution changed thrice daily. Dialysed fraction pools were frozen at -80°C and lyophilised. Lyophilised fractions were resuspended in TBS, weighed for volume determination and protein concentration assayed using a Pierce<sup>®</sup> BCA Protein Assay Kit (Thermo Fisher Scientific), as described previously (Section 3.2.1.3). Fractions were pipetted into aliquots and stored at -20°C.

#### 4.2.4.1 ELISA Analyses of Growth Factors Heparin-Fractionated DDM

Concentrations of abundant growth factors in heparin-purified DDM fractions and whole DDM were determined. Briefly, samples of each heparin-purified fraction were examined for concentrations of TGF- $\beta_1$  and BMP-2, using ELISAs as described previously (Sections 4.2.3.1 and 4.2.3.4, respectively). Total abundance of TGF- $\beta_1$  and BMP-2 in each fraction was calculated using determined volume, as described previously (Section 4.2.3.5).

## 4.2.4.2 Detection of Biglycan and Decorin in Un-fractonated DDM and FT Fraction

Aliquots of un-fractionated DDM and FT fraction were lyophilised and underwent SDS-PAGE, protein electroblotting and protein detection, as described previously (Sections 4.2.2 and 4.2.2.2-4.2.2.3). Antibodies used for protein detection are listed in Table 4.2.

Primary	Primary A	Secondary Ab	Secondary Ab	Blocking
antibody (Ab)	Dilution		dilution	peptide
Biglycan (L-15) -	1:500	Anti-goat HRP	1:50,000	Biglycan (L-15)
Santa Cruz		(sc-2020) –		Blocking
		Santa Cruz		Peptide -
				Santa Cruz
Decorin (N-15) -	1:300	Anti-goat HRP	1:50,000	Decorin (N-15)
Santa Cruz		(sc-2020) –		Blocking
		Santa Cruz		Peptide -
				Santa Cruz

Table 4.2 – Detailing antibodies and respective concentrations/blocking peptides for detection of biglycan and decorin in un-fractionated and FT of heparin-purified DDM.

#### 4.2.5 Expansion of BMMSCs Cultured with Heparin-Fractionated DDM

For subsequent analyses of biological effects of heparin-fractionated DDM, concentrations of fractions applied to BMMSCs were proportional comparative to the percentage of total recovered protein, relative to 10µg/mL of un-fractionated DDM.

Expansion of BMMSCs cultured with heparin-fractionated DDM was performed using modified protocol of the previously described thiazolyl blue tetrazolium blue (MTT) assay (Section 3.2.2). Briefly, BMMSCs (15.2-16.8 population doublings, PDs) were seeded into triplicate wells of a 96-well plate (Sarstedt) at 4x10<sup>3</sup>cells/cm<sup>2</sup> in 5% FBS (Invitrogen, UK) culture media, conditioned with heparin-fractionated DDM fractions. Cells cultured in un-conditioned media served as a control. At each time point, 20µL of media was aspirated from each well and replaced with 20µL of sterile filtered MTT solution (5mg/mL in PBS). Plates were incubated and processed, as described previously (Section 3.2.2). Experiments were performed on five separate occasions.

#### 4.2.6 Osteogenic Induction Ability of Heparin-Fractionated DDM

Osteogenic gene expression of BMMSCs over 2 and 5 days cultured heparinfractionated DDM fractions was performed, as described previously (Section 3.2.6). Briefly, BMMSCs (15.2-16.8 PDs) were seeded into 6-well or 12-well culture plates (Sarstedt) at 10<sup>4</sup> cells/cm<sup>2</sup>, in media supplemented with heparin-fractionated DDM, or basal media (negative control). RNA was extracted using an RNeasy Mini Kit (Qiagen, Germany) (Section 2.2.3), converted to cDNA (Section 2.2.4) and quantitative real time-polymerase chain reaction (q-PCR) analysis (Section 3.2.6), performed for RunX2. GAPDH was used as an internal housekeeping gene for normalisation of data. Primer sequences are detailed in Table 3.2.1. Experiments were performed on three separate occasions.

## 4.2.6.1 Mineral Deposition Analysis of BMMSCs Cultured with Heparin-Fractionated DDM

Mineral deposition of BMMSCs cultured with heparin-fractionated DDM and subsequent Alizarin red staining was performed, as described previously (Sections 3.2.5 – 3.2.5.1). Briefly, BMMSCs (15.2-16.8 PDs) were seeded into 24-well culture plates (Sarstedt) at 10<sup>4</sup> cells/cm<sup>2</sup>, in media supplemented with heparin-fractionated DDM, osteogenic induction media (positive control) or basal media (negative control); and cultured for 28 days. Cells were fixed and stained with 2% Alizarin red (Sigma-Aldrich). Stained cells were photographed under light microscopy, using a Panasonic DMC-G1 Camera. Experiments were performed on three separate occasions.

#### 4.2.6.2 Quantification of Alizarin Red Staining

Quantification of Alizarin red staining was performed, using a protocol adapted from Gregory *et al* (2004). Stained cells were incubated with  $200\mu$ L per well of 10% (v/v) acetic acid (Fisher Scientific), for 30 min at room temperature on an orbital shaker at 75rpm. Cell monolayers were transferred with the acetic acid to a 1.5mL tubes (Eppendorf). Samples were mixed by vortex for 30 s and incubated at 85°C for

10 min. Samples were cooled on ice for 5 min, followed by centrifugation at 17,000*g* for 15 min. 125µL of supernatant was transferred to a fresh 1.5mL tube (Eppendorf); and neutralised with 50µL of 10% (v/v) ammonium hydroxide (Sigma-Aldrich). 50µL of each neutralised sample was pipetted into an optically clear, white walled 96-well plate (Corning, USA) in triplicate. Absorbance at 425nm was read using a FluoSTAR OMEGA Plate Reader (BMG Labtech).

#### 4.2.7 Statistical Analyses

Statistical analyses were performed utilising One-Way ANOVA with a post-hoc Tukey test, using GraphPad InStat 3 (v3.06). Statistical values were defined as significant (\*, P<0.05), very significant (\*\*, P<0.01) or extremely significant (\*\*\*, P<0.001).

#### 4.3 Results

#### 4.3.1 Separation Profile of DDM with Silver Stain

The protein separation profile of DDM via SDS-PAGE and silver staining indicated a wide variety of protein components, ranging from around 250 kDa to 6 kDa. Three distinct protein bands were present, with apparent molecular weights of 80 kDa, 70 kDa and 50 kDa (Figure 4.1).



Figure 4.1 - Profile of proteins in DDM separated by SDS-PAGE. Three distinct bands are present at 50 kDa, 70 kDa and 80 kDa (white arrows), amongst an array of additional proteins. 10 $\mu$ g sample loaded. L = Ladder, S = Sample. Silver staining was repeated once.
#### 4.3.2 Western Blot Analyses of Growth Factors in DDM

Western blot analyses identified the presence of TGF- $\beta_1$ , BMP-2 and VEGF in DDM. Apparent molecular weights were around 50 kDa for each growth factor. Preincubation of primary antibodies used for protein blotting with respective blocking peptides resulted in an abolishment of signal, thus determining antibody specificity (Figure 4.2).



Figure 4.2 - Western blot analyses for presence of growth factors within DDM. Positive signals were identified for TGF- $\beta$ 1, BMP-2 and VEGF. However signals were abolished when incubating primary antibodies with respective blocking peptides (BP). Western blots were repeated once.

#### 4.3.3 ELISA Analyses of Growth Factor Concentrations in DDM

Quantification of a range of growth factors within DDM revealed TGF- $\beta_1$  as the most abundant factor, representing 11.3ng/mg. FGF-2, BMP-2, PDGF and VEGF demonstrated intermediate abundance at concentrations of 5.9ng/mg, 5.2ng/mg, 4.7ng/mg and 3.7ng/mg, respectively. IGF-1, BMP-4 and BMP-7 constituted the lowest abundant factors, measuring concentrations of 1.6ng/mg, 120pg/mg and 716pg/mg, respectively (Figure 4.3).



Growth factor	Abundance (ng/mg of DDM)
TGF-β <sub>1</sub>	11.3
VEGF	3.7
BMP-2	5.2
IGF-1	1.6
FGF	5.9
PDGF	4.7
BMP-4	0.12
BMP-7	0.72

Figure 4.3 - Graph and table representing the absolute concentrations of osteogenic growth factors present within DDM. Error bars represent  $\pm$ SD (n=2).

#### 4.3.4 DDM Fractionation Heparin-Affinity Chromatography

DDM was fractionated by heparin-affinity chromatography to isolate heparinbinding components. Fractions A1-A6 contained unbound constituents of DDM and was denoted flow-through (FT). Upon application of elution buffer application, a small absorption peak was observed in fractions A7-A9, followed by a smooth tailoff of absorption from fractions A10-B11. Based on the chromatogram profile, fractions A7-A9 and A10-B11 were pooled and denoted elution pool 1 (EL1) and elution pool 2 (EL2), respectively (Figure 4.4).



Figure 4.4 - Representative chromatogram for DDM separated via heparin-affinity FPLC. The initial large peak (FT) related to unbound DDM and constituted the flow through (FT) fraction. A smaller peak (EL1) was present after the increasing gradient of eluent buffer was applied, with a smooth tail off of absorbance presented up to 100% eluent buffer application (EL2). Additional FPLC runs yielded identical chromatogram profiles.

#### 4.3.4.1 Protein Recovery from Heparin-Fractionated DDM

A bicinchoninic acid (BCA) assay was performed to determine the concentrations of proteins of total loaded and heparin-purified DDM fractions, with abundance calculated from the mass of respective fractions. Relative to total DDM applied through heparin-affinity chromatography, over 95% of protein was recovered in all fractions. 79.9% of recovered protein was present in FT fraction and represents components that did not bind to heparin. EL1 contained 8.8% of total recovered protein and the remaining 6.9% was present in EL2 (Figure 4.5).



Figure 4.5 - Graph representing abundance of protein present in pre-fractionated DDM and subsequent heparin-purified DDM pools, as determined by BCA protein assay. Overall recovery of total protein from heparin-fractionation was over 95%. FT constituted 79.9% of recovered protein, whereas EL1 and EL2 contributed to 8.8% and 6.9% of recovered protein, respectively (n=1).

#### 4.3.4.2 ELISA Analyses of TGF- $\beta_1$ and BMP-2 in Heparin-Fractionated DDM

ELISA analyses of TGF- $\beta_1$  and BMP-2, growth factors known to be in high abundance in DDM, were performed to assess the degree of growth factor purification within heparin-fractionated DDM, relative to un-fractionated DDM. Neither TGF- $\beta_1$  nor BMP-2 was completely depleted from DDM via heparin-affinity chromatography. Around 75% of TGF- $\beta_1$  was depleted from DDM, with approximately 40% eluted into fraction EL2. Around 25% of BMP-2 was isolated from DDM with approximately 7% recovered in EL1 and 1.5% in EL2 (Figure 4.6).



Figure 4.6 - Graph representing the relative abundance of TGF- $\beta_1$  and BMP-2 in heparinfractionated DDM, compared to un-fractionated DDM. Around 75% of TGF- $\beta_1$  was depleted from DDM in the FT fraction, with 3% present in EL1 and 41% present in EL2. Around 25% of BMP-2 was depleted from DDM in the FT fraction, with 7% in EL1 and 1.5% in EL2 (n=1).

# 4.3.4.3 Western Blot Identification of Biglycan and Decorin in Un-Fractionated DDM and FT Fraction

Western blot analyses for the immuno-detection of biglycan and decorin were performed for un-fractionated DDM and FT fraction, to determine the potential for growth factor binding. Distinct banding was present for biglycan (BGN) and decorin (DEC) core proteins, at around 50kDa and 48kDa, respectively. Additional banding, up to 250kDa and 200kDa was detected for biglycan and decorin, respectively, indicative of glycosylated protein. Pre-incubation of the primary antibodies with respective blocking peptide (BP) resulted in abolished protein detection (Figure 4.7).





#### 4.3.5 Expansion of BMMSCs Cultured with Heparin-Fractionated DDM

After 4 days in culture, expansion of BMMSCs cultured with un-fractionated DDM was reduced, compared to un-treated cells (P<0.01). Expansion of cells cultured with FT fraction was unchanged, compared to un-fractionated DDM, however, was lower than compared with un-treated cells (P<0.05). Expansion of cells cultured with EL1 fraction was higher than cells cultured with un-fractionated DDM (P<0.01), however, was unchanged compared with un-treated cells. No change in expansion was observed for cells cultured with EL2 fraction, compared with un-fractionated DDM or with un-treated cells (Figure 4.8).



Figure 4.8 - Graph representing BMMSC expansion when cultured with heparinfractionated DDM. Cell expansion was lower for BMMSCs cultured with un-fractionated DDM and FT, fraction compared with un-treated cells. Expansion was greater for cells cultured with EL1 fraction compared with cells cultured with un-fractionated DDM. Error bars represent ±SD (n=5). \* = P < 0.05, \*\* = P < 0.01, \*\*\* = P < 0.001.

#### 4.3.6 RunX2 Expression by BMMSCs Cultured with Heparin-Fractionated DDM

After 2 days in culture, RunX2 gene expression was down-regulated in BMMSCs cultured with FT fraction relative to un-fractionated DDM (P<0.05). RunX2 expression of BMMSCs was not changed when cultured in any DDM fraction relative to un-treated cells. After 5 days, RunX2 expression was up-regulated for BMMSCs cultured in un-fractionated DDM, relative to cells cultured with FT (P <0.05), EL1 (P < 0.01) and EL2 (P < 0.01) fractions, in addition to un-treated cells (P < 0.01). FT, EL1 and EL2 had no effect on RunX2 expression, compared with un-treated BMMSCs (P > 0.05) (Figure 4.9).



Figure 4.9 - Expression of RunX2 in BMMSCs cultured with heparin-fractionated DDM after 2 and 5 days. No DDM fraction provoked an up-regulation of RunX2, compared with untreated cells after 2 days. However, un-fractionated DDM significantly up-regulated RunX2, expression relative to all other test groups after 5 days. Error bars represent ±SD (n=3). \* = P < 0.05, \*\* = P < 0.01.

#### 4.3.7 Alizarin Red Staining of BMMSCs Cultured with Heparin-Fractionated DDM

After 28 days in culture, BMMSCs cultured with heparin-fractionated DDM were fixed and stained with Alizarin red, to highlight mineral deposition. BMMSCs cultured with un-fractionated DDM deposited small and sparse mineralised deposits. No prominent mineralised nodules were observed for cells cultured with FT, EL1 or EL2 fractions, or un-treated cells. BMMSCs cultured in osteogenic media produce numerous and large mineralised nodules, which stain readily with Alizarin red (Figure 4.10).



Figure 4.10 - Micrograph images of BMMSCs stained by Alizarin red for mineral deposition analysis after 28 days in culture. BMMSCs cultured with un-fractionated DDM produce scarce mineralisation (white arrow). No visible mineralised nodule staining is evident for BMMSCs cultured with FT, EL1 or EL2 fractions, or un-treated cells. White bars represent 100µm.

#### 4.3.7.1 Quantification of Alizarin Red Staining

As a more sensitive method of determining the degree of Alizarin red staining of BMMSCs cultured with DDM fractions, the stain was quantified using spectroscopy. Alizarin red staining was up-regulated for BMMSCs cultured with un-fractionated DDM compared with BMMSCs cultured with all heparin-fractionated DDM (P<0.001). Increased Alizarin red staining was identified in FT (P<0.05), EL1 (P<0.05) and EL2 (P<0.001) treated BMMSCs compared to un-treated cells (Figure 4.11).



Figure 4.11 - Graph representing intensity of Alizarin red staining of BMMSCs cultured with DDM fractions for 28 days. Error bars represent  $\pm$ SD (n=3). \* = P < 0.05, \*\*\* = P < 0.001.

#### 4.4 Discussion

This Chapter has elucidated the growth factor profile of DDM, by revealing the concentrations of these factors, including TGF- $\beta_1$ , BMP-2 and VEGF. A pertinent finding of this Chapter was that fractionation of growth factors via heparin-affinity chromatography reduced their potency compared to un-fractionated DDM; therefore, suggesting that interactions of growth factors with other matrix components in DDM are essential for osteogenic potency towards BMMSCs.

SDS-PAGE and silver stain analysis of DDM in this Thesis showed a wide range of proteins with a highly prominent band at around 70kDa; and two further yet slightly less distinct bands at 50kDa and 80kDa. The band present at 70kDa is likely to be albumin, as this has been suggested to be sequestered within dentine (Thomas and Leaver, 1975); and is indicated to constitute a high abundance relative to other proteins in DDM (Park et al, 2009; Jágr et al, 2012). The presence of a band at 50kDa may represent growth factors, in addition to the core proteins of the small, leucine-rich proteoglycans (SLRPs), biglycan and decorin, as these have pertinent roles in directing dentine formation and mineralisation (Embery et al, 2001; Haruyama et al, 2009). Literature reports indicate that dentine constitutes a vast array of various protein components, noted as 233 for ethylenediamine tetra-acetic acid (EDTA) (Park et al, 2009) and 289 for EDTA/guanidium hydrochloride extracted dentine (Jágr et al, 2012). Amongst candidates for proteins within EDTA extracted DDM in this Thesis,  $\alpha$ 2-HS glycoprotein (also known as fetuin) is acknowledged as a highly abundant protein within dentine (Jágr *et al*, 2012). Fetuin is a glycoprotein of around 55kDa (To et al, 1995) and is regarded as a regulator of mineralisation (Schafer et al, 2003); and is implicated in inhibition of the growth of hydroxyapatite crystals outside of collagen fibrils (Price et al, 2009). Collagenous proteins constitute around 85-90% of total dentine proteins (Linde, 1989). Type I collagen is the most predominant, with a lesser abundance of collagen types III, V and VII (Garant, 2003), regarded as structural proteins required for the deposition of hydroxyapatite crystals (Marshall et al, 1997). Due to the preparative protocols utilised in this Thesis, fibrillar collagenous proteins are likely to largely be excluded from DDM due to their insolubility, however soluble collagens may be present. The

remaining non-collagenous components of dentine represent a large number of proteins, with diverse roles including those involved with mineralisation, extracellular matrix (ECM) organisation, inflammation, immune response and cell signalling (Park *et al*, 2009, Smith *et al*, 2012).

TGF- $\beta_1$  was identified in DDM as an approximate 52kDa protein, in addition to being the most abundant growth factor analysed, at 11.3ng/mg. Unprocessed pre-cursors of TGF- $\beta_1$  are reported to run at apparent molecular weights of 44-56kDa on SDS-PAGE (Gentry et al, 1988), however, isoforms of 50kDa are still biologically active (Baillie *et al*, 1996). TGF- $\beta$ s are secreted as latent-precursors containing a latencyassociated peptide (LAP) region, which requires proteolytic cleavage or physical interactions with other proteins to enable release of bioactive TGF- $\beta_1$  (Verrecchia and Mauviel, 2002). TGF- $\beta_1$  has been widely investigated in dental tissues and possesses well-acknowledged roles in stimulating migration (Howard et al, 2010), proliferation (Melin et al, 2000; Nie et al, 2006), ECM production and differentiation (Melin et al, 2000; Nie et al, 2006; He et al, 2008) of dental pulp-derived cells. Literary reports indicate that TGF- $\beta_1$  is one the most abundant cytokines in human bone matrix at around 200ng/g (Hering *et al*, 2001). TGF- $\beta_1$  does not solely induce osteogenic differentiation of mesenchymal stem cells (MSCs) (Kutagiri et al, 1994), however, it can indirectly influence osteogenesis by enhancing expression of BMP-1 (Lee et al, 1997), which is required for the generation of mature collagen fibrils in the formation of bone (Asharani *et al*, 2012). TGF- $\beta_1$  can also induce migration (Tang et al, 2009) and proliferation (Zhang et al, 2015a) of BMMSCs.

BMP-2 was identified in DDM as a 50 kDa protein as determined by Western blotting, additionally determined by ELISA assay to be present at the concentration of 5.2ng/mg. BMP-2 is synthesised as a large inactive precursor of around 45kDa which is proteolytically cleaved to generate mature BMP-2 monomer (Roedel *et al*, 2013). It is widely acknowledged that BMP-2 is a potent growth factor for driving osteogenesis of MSCs. Application of exogenous BMP-2 increases mRNA expression of RunX2 in human BMMSCs (Gori *et al*, 1999) and additionally promotes increased mRNA expression of osteocalcin (OC) and subsequent mineral deposition in BMMSCs (Fromigué *et al*, 1998). Lecanda *et al* (1997) report that exogenous BMP-2

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applied to human BMMSCs enhances mRNA expression and protein synthesis of osteopontin (OPN), OC and collagen type I. Application of rhBMP-2 to human dental pulp cells enhances gene expression of dentine sialophosphoprotein (DSPP) (Saito *et al*, 2004). Blockage of BMP receptors in stem cells from human exfoliated, deciduous teeth (SHED) abolishes expression of DSPP and dentine matrix protein-1 (DMP-1) (Casagrande *et al*, 2010). Furthermore, rhBMP-2 has been demonstrated to induce proliferation and migration of periodontal ligament (PDL)-derived cells, accompanied with enhanced cementum formation in a denuded PDL model in rats (King and Hughes, 2001).

VEGF was detected in DDM at as an approximate 50 kDa protein via Western blotting and further determined to be present in DDM via ELISA at 3.7ng/mg. The existence of an inactive 47kDa isoform of VEGF is reported and requires proteolytic activation to induce bioactivity (Tee and Jaffe, 2001). VEGF is implicated in driving angiogenesis, to enhance fracture repair (Kaigler *et al*, 2013). VEGF is additionally associated with increasing the density of micro-vessels in severed human dental pulps *in vitro* (Mullane *et al*, 2008). Culture of human dental pulp-derived, stem cells (DPSCs) in osteogenic induction medium supplemented with VEGF enhances calcium deposition (D' Alimonte *et al*, 2011).

Although Western blots indicated the presence of a few growth factors in DDM, ELISA analyses provided further data relating to the presence of additional growth factors, in addition to their relative concentrations in DDM, thereby uncovering the ratios of these factors relative to each other. FGF-2 and PDGF were identified in DDM at relatively intermediate concentrations, compared to IGF-1, BMP-4 and BMP-7, which were identified as being present in lower concentrations.

FGF-2 has been demonstrated to extend *ex vivo* expansive capacity and maintain osteogenic precursors of human MSCs. FGF-2 is also reported to stimulate proliferation (Tsutsumi *et al*, 2001; Ahn *et al*, 2009) and migratory behaviour (Schmidt *et al*, 2006; Latifi-Pupovici *et al*, 2015) of MSCs *in vitro*. FGF-2 promotes the proliferation and migration of human dental pulp-derived cells; however, formation of calcified nodules is attenuated (Shimabukuro *et al*, 2009).

Furthermore, FGF-2 enhances proliferation and suppresses RunX2, type I collagen and calcium deposition in stromal cells, derived from the PDL (Lee *et al*, 2012b).

PDGF is suggested to not possess a direct role in the differentiation of human MSCs towards an osteoblastic lineage, as inhibition of receptors does not negatively affect staining for mineral deposition or OC for cells treated with osteogenic induction medium (Kumar *et al*, 2010). PDGF has been demonstrated to elicit chemotactic responses of fetal rat calvarial cells *in vitro* (Hughes *et al*, 1992). PDGF is also reported to counter bone resorption during fracture healing by enhancing osteoprotegerin (OPG) production (McCarthy *et al*, 2009). In the dental pulp, PDGF enhances proliferation of human dental pulp-derived, fibroblasts (Rutherford *et al*, 1992).

IGF-1 may aid in acceleration of migratory activity by up-regulating CXCR4 on membranes of MSCs (Huang *et al*, 2012b). Both IGF-1 and IGF-1 receptor (IGF-1R) are expressed in human dental pulps, however, higher expression is noted for teeth with fully developed roots and therefore, has postulated roles in the differentiation and subsequent mineralisation of dental-derived, progenitor cells (Caviedes-Bucheli *et al*, 2004; 2007).

The BMP family of growth factors exert effects on MSCs that induce osteogenic differentiation. Application of BMP-4 to human BMMSCs increases expression of collagen type I mRNA (Lecanda *et al*, 1997). Presentation of BMP-4 to canine dental pulps promotes the formation of reparative dentine (Nakashima *et al*, 1994). Furthermore, BMP-7 induces mineralisation of exposed rat pulp molars (Six *et al*, 2002). Used in conjunction with osteogenic induction media, BMP-7 enhances upregulation of osterix (Osx) and OC of human BMMSCs (Shen *et al*, 2010). When applied together, BMP-4 and BMP-7 additionally modulate osteogenic differentiation of human MSCs and provoke mineralised matrix deposition (Lavery *et al*, 2008).

Although the growth factors identified in DDM exert varying effects on MSCs, they may be potentially acting in synergy to exert various biological responses of BMMSCs (as described in Chapter 3). To determine the role of growth factors in the

osteogenic potency of DDM, heparin-affinity chromatography was performed with the aim of purifying growth factors from the matrix. Within this study, heparinaffinity resulted in partial depletion of the assessed growth factors TGF- $\beta_1$  and BMP-2 from DDM. Native TGF- $\beta_1$  is reported to completely bind to heparinsepharose, which is eluted in 0.9-1.2M NaCl (McCaffrey *et al*, 1992). BMP-2 also contains a heparin-binding site with elution from heparin-sepharose achievable with 0.7M NaCl (Ruppert *et al*, 1996). Around 75% of TGF- $\beta_1$  was depleted from DDM, with 40% recovered in EL2 fraction and around 25% remaining in the flow through and only 3% present in EL1. Conversely, only 25% of BMP-2 was depleted from DDM, with 7% recovered in EL1 fraction and just over 1% in EL2. For both growth factors analysed, full depletion was not achieved despite both factors containing heparin-binding sites, thus indicating variation with heparin-binding affinity potentially due to inhibition of other matrix components present in DDM.

Biglycan and decorin are reported to be fundamental proteins in dentine matrix with proposed roles in sequestration of growth factors and prevention of overactivation, notably TGF- $\beta_1$  (Yamaguchi *et al*, 1990; Hildebrand *et al*, 1994). Furthermore, biglycan reportedly binds to BMP-2 (Mochida *et al*, 2006). Western blot analysis of biglycan and decorin within the FT fraction identified the presence of these SLRPs, thereby suggesting these may be competitively inhibiting binding of TGF- $\beta_1$  and BMP-2 to heparin and thus, are unable to be wholly isolated from DDM. Due to limited sample availability, the presence of additional growth factors in heparin-purification fractions was not analysed. PDGF-BB and FGF-2 both additionally bind to heparin-sepharose and are eluted at 0.5-0.6M NaCl (Mangrulkar *et al*, 1995) and 1.6-1.8M NaCl (Gospodarowicz *et al*, 1984), respectively. Consequently, any bound PDGF-BB and FGF-2 were likely to be eluted in the EL2 fraction, therefore suggesting EL2 to be a richer source of isolated growth factors from DDM relative to EL1.

Whilst expansion of BMMSCs was reduced when cultured with un-fractionated DDM and FT fraction, compared with un-treated cells, EL1 and EL2 fractions did not change cell expansion over 4 days, compared to un-treated cells. To further determine the bioactivity of heparin-fractionated DDM, expression of RunX2 in

BMMSCs cultured with DDM fractions was assessed. Un-fractionated DDM provoked an up-regulation of RunX2 gene expression after 5 days, compared to untreated cells, concurrent with previous results (Section 3.3.4). Heparin-fractionation of DDM resulted in abolished up-regulation of RunX2 gene expression, compared to un-treated cells. Subsequently, mineralised matrix deposition of BMMSCs treated with heparin-fractionated DDM was diminished, compared to matrix deposition of cells treated with un-fractionated DDM. In contrast, heparin-binding components of EDTA extracted DDM is capable of stimulating reactionary dentinogenesis, following implantation into teeth (Smith *et al*,1995).

Relative to un-fractionated DDM, FT fraction was depleted of both BMP-2 and TGF- $\beta_1$ , however, a greater percentage of depletion was evident for TGF- $\beta_1$ . Even though growth factor combinations of varying concentrations and ratios were determined in DDM fractions, osteogenic potency was attenuated compared to un-fractionated DDM, therefore indicating the importance of optimal matrix composition for potentiating osteogenesis of BMMSCs. TGF- $\beta_1$  can synergistically act with BMP-2 to enhance bone formation by around 5-fold compared to BMP-2 alone (Tachi et al, 2011) and therefore, depletion of TGF- $\beta_1$  from the FT fraction may be responsible for diminished differentiation capacity, compared to whole DDM. Although not explored, several other heparin-binding components may additionally have been depleted from DDM and thus, potentially reduced their concentration within the FT fraction. TGF- $\beta_1$ , FGF-2 and PDGF-AB increase expression of BMP receptor-1B (BMPR-1B) in human bone cells, which augments cellular responses to BMP-2 via Smad 1/5/8 phosphorylation (Singhatanadgit et al, 2006). BMP-2 induced bone formation can also be synergistically enhanced by exogenous VEGF application, however, high proportions of VEGF: BMP-2 results in decreased synergism (Peng et al, 2005). Combination of rhBMP-2 and dentine sialoprotein (DSP) synergistically enhances OC gene expression and mineral deposition of cementoblasts, compared to singular application (Lee et al, 2014).

Although growth factors were depleted from DDM and were recovered in subsequent elution fractions, neither recovered fraction elicited the extent of osteogenic induction relative to un-fractionated matrix. Concentrations of both TGF- $\beta_1$  and BMP-2 were greater than 10-fold smaller in fraction EL1, relative to unfractionated DDM. Therefore, EL1 treated BMMSCs may not have been exposed to concentrations of growth factors high enough to induce osteogenic effect, due to the dose-dependent response of DDM on BMMSCs (see Chapter 3 for details). TGF- $\beta_1$  was predominantly present within fraction EL2, relative to BMP-2. However, TGF- $\beta_1$  does not exert osteogenic potency towards BMMSCs (Katagiri *et al*, 1994). RunX2 expression can be repressed by TGF- $\beta_1$  and thus, inhibit osteoblastic differentiation (Alliston *et al*, 2001), with differentiation potential restored following TGF- $\beta_1$  inhibition (Takeuchi *et al*, 2010). Modulation of TGF-  $\beta_1$  bioactivity by sequestration by biglycan and decorin is acknowledged (Hildebrand *et al*, 1994; Baker *et al*, 2009). Analysis of SLRPs in elution fractions would have been beneficial to determine the nature of TGF- $\beta_1$ , with regards to activity regulation by biglycan and/or decorin. However, limited sample availability prevented this investigation.

In conclusion, DDM contains a plethora of different protein components amongst which are a range of growth factors with various roles in directing osteogenesis. The low concentration of growth factors present within DDM alludes to synergistic action to stimulate biological responses. Purification of DDM via heparin-affinity chromatography diminishes osteogenic induction capability relative to unfractionated matrix, therefore suggesting that DDM presents a 'cocktail' of bioactive factors with optimal biological ratios to exert osteogenic effect.

# Chapter 5 - Determination of the Significance of Biglycan and Decorin for the Osteogenic Potency of Demineralised Dentine Matrix (DDM)

#### 5.1 Introduction

Biglycan and decorin belong to the small, leucine-rich proteoglycan (SLRP) family and consist of protein cores of around 45 kDa, associated with two or one attached glycosaminoglycan (GAG) chains, respectively (Embery *et al*, 2001; Seidler *et al*, 2006). Both biglycan and decorin are ubiquitously expressed in a variety of tissues, including bone, kidneys, eyes and skin (Bianco *et al*, 1990). Models of SLRP knockout (KO) are associated in a variety of diseases such as osteoporosis, osteoarthritis and Ehler's-Danlos syndrome (Ameye *et al*, 2002). As both biglycan and decorin in bone (Ingram *et al*, 1993), KO models for these SLRPs in rodents have determined their importance in maintenance of skeletal tissues. In mouse KO models, the absence of decorin does not affect skeletal mass (Corsi *et al*, 2002). However, dual KO of both biglycan and decorin results in marked osteopenia. The KO of biglycan alone results in viable animals at birth, with no apparent bone defects although mild osteopenia is noted for aged mice (Xu *et al*, 1998). Both biglycan and decorin are also present within dentine and are proposed to serve roles in the regulation of cell proliferation and matrix mineralisation (Waddington *et al*, 2003b).

Although regarded as pertinent constituents of mineralised tissues, SLRPs are additionally implicated in the binding and modulation of growth and signalling factors. Both biglycan and decorin are reported to bind to type I collagen and transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) with a postulated role in the sequestration of TGF- $\beta_1$  to the extracellular matrix (ECM) (Hildebrand *et al*, 1994). Biglycan can enhance bone morphogenetic protein-4 (BMP-4) induced RunX2 expression by increasing of BMP-4 binding to its receptor (Chen *et al*, 2004). Biglycan can also bind bone morphogenetic protein-2 (BMP-2) and its receptor to promote osteogenic differentiation (Mochida *et al*, 2006). Decorin is able to enhance responses to BMP-2 (Moreno *et al*, 2005; Gutierrez *et al*, 2006). Additionally, biglycan can interact with Wnt3a ligand and its receptor LRP6, to enhance canonical

Wnt signalling in osteogenesis (Berendsen *et al*, 2011). Therefore, SLRPs are obtaining increasing recognition as important mediators in modulation of cell signalling events both with and without associated factors.

Whilst numerous studies have explored the importance of biglycan and decorin in rodent tissues and cells, comparatively few studies are currently available that investigates the effects of these SLRPs on human bone marrow-derived, mesenchymal stem cells (BMMSCs) and their influence in osteogenesis. This Chapter aimed to elucidate the importance of biglycan and decorin in the osteogenic potency of demineralised dentine matrix (DDM) towards human BMMSCs.

#### 5.2 Materials and Methods

#### 5.2.1 Immunoprecipitation (IP) of Biglycan and Decorin from DDM

DDM was obtained from human teeth, as previously described (Section 3.2.1.2). Immunoprecipitation (IP) of biglycan and decorin from DDM was performed using a Pierce<sup>®</sup> Crosslink IP Kit (Thermo Fisher Scientific, USA), according to manufacturer's protocol. All steps were performed at 4°C, unless otherwise stated; and all centrifugation performed at 1,000*g* for 1 min. IP was performed in separate spin columns for each protein to be isolated. Both individual and dual depletion of biglycan and decorin from DDM was performed.

20μL of Pierce Protein AG Plus Agarose was pipetted into a Spin Column, centrifuged and flow-through was discarded. Resin was washed twice with 100µL of Couple Buffer. The base of the column was tapped onto a clean paper towel and sealed with a bottom plug. 10µg of biglycan and decorin antibodies (as detailed in Table 5.1) were added to 5µL of 20x Couple Buffer and total volume adjusted to 100 $\mu$ L with 18.2M $\Omega$  H<sub>2</sub>O. Solutions of biglycan and decorin antibodies were added to resin-containing spin columns, which were sealed and incubated at room temperature for 1 h, with gentle agitation. Columns were centrifuged and washed once with  $100\mu$ L and twice with  $300\mu$ L of Couple Buffer, with each flow through discarded. 2.5µL of 20x Couple Buffer, 9µL of 2.5mM disuccinyl suberate (DSS) and 38.5 $\mu$ L of 18.2M $\Omega$  H<sub>2</sub>O was added to each spin column, followed by incubation at room temperature for 1 h, with gentle agitation. Columns were centrifuged, followed by addition of 50µL of Elution Buffer, and centrifugation again. Columns were washed twice with  $100\mu$ L of Elution Buffer followed by washing twice with 200µL of IP Lysis/Wash Buffer. Columns were sealed, stored in 200µL of IP Lysis/Wash Buffer and stored at 4°C until required.

For pre-clearing,  $80\mu$ L of Control Agarose Resin Slurry ( $40\mu$ L of settled resin) was pipetted into fresh spin columns, centrifuged and washed with  $100\mu$ L of Couple Buffer. DDM was reconstituted in tris buffered saline (TBS) to an anticipated concentration of 1mg/mL and sterile filtered through a  $0.22\mu$ M syringe filter (Millipore, USA); and final volumes adjusted to 1.2mL with TBS. Protein concentrations were determined using a Pierce<sup>®</sup> BCA Protein Assay Kit (Thermo Fisher Scientific), as described previously (Section 3.2.1.3). 600µL of DDM solution was added to each column containing 40µL of settled resin, the columns were sealed and incubated for 30 min, with gentle agitation. Spin columns were centrifuged and flow through retained for the IP procedure.

Columns containing immobilised antibodies were washed twice with  $100\mu$ L of TBS and sealed with a bottom plug.  $600\mu$ L of DDM solution at  $500-1000\mu$ g/mL was added to each spin column, the column sealed and incubated overnight at 4°C with gentle agitation. DDM solution was eluted and columns were washed thrice with  $200\mu$ L of TBS, followed by one wash of  $100\mu$ L of Conditioning Buffer. Columns were placed into a 1.5mL collection tube containing  $5\mu$ L of 1M tris base (pH 9.5) (Fisher Scientific, UK),  $10\mu$ L of Elution Buffer added to each column and centrifuged. Columns were retained in collection tubes,  $50\mu$ L of Elution Buffer added to the resin and incubated for 5 min at room temperature. Columns were centrifuged and flow through retained. Elution steps were repeated into a fresh collection tube and elution volumes for each isolated protein pooled.

Depleted flow-through fractions of DDM were weighed for volume determination. Volumes for biglycan and decorin depleted flow through and enriched elution pools utilised in forthcoming experiments were proportional, relative to the total volume of depleted elution.

For dual-depletion, the above steps were performed, however, DDM was sequentially depleted of biglycan, followed by decorin.

## 5.2.1.1 Protein Separation Using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Protein Electroblotting

Fractions from the IP procedure were assayed to determine depletion and subsequent recovery of biglycan and decorin from DDM, in addition to co-depleted TGF- $\beta_1$ , as previously described (Sections 4.2.2 and 4.2.2.2). Briefly, IP fractions were lyophilised and reconstituted in 1x Laemmli Sample Buffer to a concentration relative to  $2\mu g/\mu L$  of un-depleted DDM. Samples were heated to 95°C for 5 min and 15 $\mu L$  loaded into 4-15% Mini Protean TGX pre-cast gels (BioRad Laboratories, USA),

alongside 7.5µL of Kaleidoscope Pre-Stained Protein Standards (BioRad Laboratories). Gels were placed in an electrophoresis cell with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) running buffer (25mM trisbase (Sigma-Aldrich, UK), 192mM glycine (Fisher Scientific), 0.1% SDS (Fisher Scientific), pH 8.3) and separated by electrophoresis at 200V for 30-35 min, until the migration front reached the bottom of the gel.

Gels were soaked in transfer buffer (25mM tris-base, 192mM glycine, 20% (v/v) methanol (Fisher Scientific), pH 8.3); and proteins transferred onto nitrocellulose by electroblotting.

#### 5.2.1.2 Immunoblotting for Protein Detection

Detections of proteins were performed, as previously described (Section 4.2.2.3). Briefly, membranes were blocked in 5% (w/v) milk (Tesco, UK) in TBS/0.05% Tween-20 (Sigma-Aldrich) (TBS-T) for 1 h at room temperature, followed by incubation with primary antibodies overnight at 4°C. Membranes were washed four times with TBS-T and incubated with respective secondary antibodies for 1 h at room temperature. Membranes were washed four times in TBS-T and a further twice in TBS. Membranes were treated with ECL Reagent (GE Healthcare, UK) and exposed to X-ray development film (GE Healthcare). Details of antibodies used are shown in Table 5.1.

Primary antibody	Primary Ab dilution	Secondary Ab	Secondary Ab
(Ab)			dilution
Biglycan (L-15) –	1:500	Anti-goat HRP (sc-	1:50,000
Santa Cruz (USA)		2020) – Santa Cruz	
Decorin (N-15) –	1:300	Anti-goat HRP (sc-	1:50,000
Santa Cruz		2020) – Santa Cruz	
TGF- $\beta_1$ (V) – Santa	1:200	Anti-rabbit HRP (sc-	1:25,000
Cruz		2030) – Santa Cruz	

Table 5.1 – Detailing antibodies used in IP and respective dilutions used for protein detection of IP fractions.

#### 5.2.2 Expansion of BMMSCs Cultured with DDM IP Fractions

Expansion of BMMSCs cultured with DDM IP fractions were performed over 4 days, using modified protocol of the previously described thiazolyl blue tetrazolium blue (MTT) assay (Section 3.2.2). Briefly, BMMSCs (16-17.5 population doublings, PDs) were seeded into triplicate wells of a 96-well plate (Sarstedt, UK) at 4x10<sup>3</sup>cells/cm<sup>2</sup>, in 5% FBS (Invitrogen, UK) culture media conditioned with un-depleted, SLRP-depleted and SLRP-enriched IP fractions. Cells cultured in un-conditioned media served as a control. At each time point 20µL, of media was aspirated from each well and replaced with 20µL of sterile filtered MTT solution (5mg/mL in PBS) (Sigma-Aldrich). Plates were incubated and processed, as described previously (Section 3.2.2). Experiments were performed on three separate occasions.

#### 5.2.3 Osteogenic Induction Ability of DDM IP fractions

Osteogenic gene expressions by BMMSCs over 2, 5 (individual and dual depleted) and 28 (individual depletion only) days cultured with DDM IP fractions were performed, as described previously (Section 3.2.6). Briefly, BMMSCs (16-17.5 PDs) were seeded into 6-well or 12-well culture plates (Sarstedt) at 10<sup>4</sup> cells/cm<sup>2</sup>, in media conditioned with IP DDM fractions or un-conditioned media (negative control). RNA was extracted using an RNeasy Mini Kit (Qiagen, Germany) (Section

2.2.3), converted to cDNA (section 2.2.4.) and quantitative real time-polymerase chain reaction (q-PCR) analysis performed for RunX2 (days 2 and 5) and osteocalcin (OC) (day 28) gene expression (section 3.2.6). GAPDH was used as an internal housekeeping gene for normalisation of data. Primer sequences are detailed in Table 5.2. Experiments were performed on three separate occasions.

Gene	Primer sequence: 5'-3'	Annealing	Product	Primer
product		temperature	length	designer
		(°C)	(bp)	
GAPDH	F: TTCTTTTGCGTCGCCAGCCGA	55	96	Author
	R: GTGACCAGGCGCCCAATACGA			
RunX2	F: GGTTAATCTCCGCAGGTCACT	55	80	Author
	R: CCCTCTGTTGTAAATACTGCTTGC			
OC	F: GGCAGCGAGGTAGTGAAGAG	55	102	Dr. Wayne
	R: CTCACACACCTCCCTCCT			Ayre (Cardiff
				University)

Table 5.2 – Details of primer sequences used for q-PCR analysis of genes expressed by BMMSCs during culture with SLRP IP fractions.

#### 5.2.3.1 Mineral Deposition Analysis of BMMSCs Cultured with DDM IP fractions

Alizarin red staining of BMMSCs cultured with DDM IP fractions was performed, as described previously (Sections 3.2.5.1 - 3.2.5.2). Briefly, BMMSCs (16-17 PDs) were seeded into 24-well culture plates (Sarstedt) at  $10^4$  cells/cm<sup>2</sup>, in media conditioned with DDM IP fractions or un-conditioned media (negative control). Cells were cultured for 28 days, with media changes performed every 2 days. Cells were washed, fixed with 10% v/v formaldehyde (Sigma-Aldrich) and stained with 2% Alizarin red (Sigma-Aldrich). Stained cells were washed with deionised distilled H<sub>2</sub>O (ddH<sub>2</sub>O), air-dried and photographed under light microscopy, using a Panasonic DMC-G1 Camera. Experiments were performed on three separate occasions.

#### 5.2.3.2 Quantification of Alizarin Red Staining

Quantification of Alizarin red staining was performed, as described previously (Section 4.2.6.2).

#### 5.2.4 Statistical Analyses

Statistical analyses were performed utilising One-Way ANOVA with a post-hoc Tukey test, using GraphPad InStat 3 (v3.06). Statistical values were defined as significant (\*, P<0.05), very significant (\*\*, P<0.01) or extremely significant (\*\*\*, P<0.001).

#### 5.3 Results

#### 5.3.1 Conformation by Western blot of IP of Biglycan and Decorin from DDM

Biglycan and decorin, present in DDM, were successfully depleted and recovered using immunoprecipitation. For both biglycan, banding at around 50 kDa was detected via Western blot in un-depleted DDM and respective enriched-DDM, indicative of core protein. For decorin, banding at around 48kDa was observed in un-depleted DDM and respective enriched-DDM. For respective enriched DDM fractions of biglycan and decorin, additional bands are noted up to around 150 kDa, indicative of glycosylated core proteins. No bands were detected in depleted matrices and thus, confirmed depletions (Figure 5.1).



Figure 5.1 – Western blot analyses of DDM for immuno-depletion of biglycan and decorin for individual depletion (top) and dual depletion (bottom). Core proteins of 50 kDa (biglycan) and 48kDa (decorin) were present in un-depleted DDM, which are not present in depleted matrix. Respective bands, in additional to bands indicative of glycosylated proteins in the enriched matrices indicate the recovery of immuno-depleted proteins. Western blots were repeated once.

#### 5.3.2 Western Blot Determination of TGF- $\beta_1$ Co-Depletion from DDM

Western blot analysis for the immuno-detection of TGF- $\beta_1$  was performed on immunoprecipitation fractions of DDM to detect co-precipitation. TGF- $\beta_1$  was present in DDM at around 50 kDa, as determined previously (Section 4.3.2). Banding was not present in DDM depleted of biglycan or decorin, however, bands were present in fractions of respective elution pools (Figure 5.2).



Figure 5.2 - Western blot for TGF- $\beta_1$  presence in individual immunoprecipitation fractions. Bands at around 50 kDa were present in un-depleted DDM, biglycan-enriched and decorinenriched fractions. No bands are detectable in biglycan- and decorin-depleted, DDM fractions.

# 5.3.3 Expansion of BMMSCs Cultured with Individually Immuno-Precipitated DDM Fractions

Over 96 h in culture, expansion of BMMSCs cultured with un-depleted DDM was reduced after 48 h, compared to un-treated cells (P<0.01), however this reduction was not significant after 96 h. Expansion of cells cultured with biglycan-depleted DDM was not changed compared to cells cultured with un-depleted DDM, or un-treated cells. After 72 h in culture, expansion of cells cultured with decorin-enriched DDM was higher compared to un-treated cells (P<0.01), whereas cell expansion when cultured with biglycan-enriched DDM was significantly higher than un-treated cells after 96 h (P<0.05). Expansion of cells cultured with decorin-depleted DDM was not changed compared to un-treated cells over 96 h (Figure 5.3).



Figure 5.3 - Graph representing BMMSC expansion when cultured with individual immunodepletion DDM fractions. After 96 h, culture of cells with biglycan- and decorin-enriched DDM resulted in enhanced cell expansion compared with cells cultured with un-depleted DDM, in addition to un-treated cells. Error bars represent ±SD (n=3). \* = P<0.05, \*\* = P<0.01, \*\*\* = P<0.001.

### 5.3.4 Expansion of BMMSCs Cultured with Dual Immuno-Precipitated DDM Fractions

After 96 h in culture, expansion of BMMSCs cultured with un-depleted and BGN/DEC-depleted DDM was significantly reduced compared to un-treated cells (P<0.05). Cell expansion for BMMSCs cultured with biglycan/decorin-enriched DDM was significantly higher than for cells cultured with un-depleted (P<0.01) and biglycan/decorin-depleted (P<0.05), DDM. BMMSC expansion was not changed when cultured with biglycan/decorin-enriched DDM, compared with un-treated cells. No differences in cell expansion were observed between experimental groups up to 72 h in culture (Figure 5.4).



Figure 5.4 - Graph representing BMMSC expansion when cultured with dual immunodepletion DDM fractions. BMMSCs cultured with un-depleted and biglycan/decorindepleted DDM was reduced, compared to un-treated cells after 96 h. However, expansion was not affected by culturing with biglycan/decorin enriched DDM, compared to untreated cells. Error bars represent  $\pm$ SD (n=3). \* = P<0.05, \*\* = P<0.01.

### 5.3.5 RunX2 Expression of BMMSCs cultured with Individual Immuno-Precipitated DDM Fractions

After 2 days in culture, RunX2 gene expression was down-regulated in BMMSCs cultured with decorin-enriched DDM, compared with cells cultured with all other DDM IP fractions, except for decorin-depleted DDM. After 5 days in culture, only BMMSCs cultured with decorin-depleted DDM demonstrated statistically significantly higher RunX2 expression, compared with un-treated cells (P<0.05). RunX2 expression was significantly lower for cells treated with biglycan-depleted DDM, compared to un-depleted (P<0.05) and decorin-depleted (P<0.01), DDM fractions (Figure 5.5).



Figure 5.5 - Expression of RunX2 in BMMSCs cultured with individually immuno-depleted DDM fractions after 2 and 5 days. Culture of cells with decorin-enriched DDM resulted in a reduction of RunX2 expression, compared to all other groups after 2 days. After 5 days in culture, decorin-depleted DDM resulted in the highest expression of RunX2 in BMMSCs, whereas treatment of cells with decorin-enriched DDM resulted in the lowest RunX2 expression. Error bars represent  $\pm$ SD (n=3). \* = P<0.05, \*\*\* = P<0.001.

# 5.3.6 RunX2 Expression of BMMSCs Cultured with Dual Immuno-Precipitated DDM Fractions

After 2 days in culture, there were no differences in RunX2 gene expression between all of the groups analysed. After 5 days in culture, there were additionally no distinct changes in RunX2 gene expression, although cells cultured with un-fractionated DDM demonstrated the highest level of expression (Figure 5.6).



Figure 5.6 - Expression of RunX2 in BMMSCs cultured with dual immuno-depleted DDM fractions after 2 and 5 days. No statistically significant differences for gene expression were observed at the two time-points (P>0.05). Error bars represent  $\pm$ SD (n=3).

### 5.3.7 Osteocalcin (OC) Expression of BMMSCs Cultured with Individual Immuno-Precipitated DDM Fractions

After 28 days in culture, there was no difference in expression of OC in BMMSCs treated with un-depleted DDM, compared to biglycan-depleted DDM and decorindepleted DDM. Whilst OC expression appeared to be lower for BMMSCs in other treatment groups, compared to un-depleted DDM, only cells cultured with decorinenriched DDM demonstrated statistically significantly lower OC expression, compared to un-depleted DDM (P<0.05). BMMSCs cultured in decorin-depleted DDM demonstrated significantly greater OC expression, compared to un-treated cells (P<0.01) (Figure 5.7).



Figure 5.7 - Expression of OC in BMMSCs cultured with individual immuno-depleted DDM fractions after 28 days. Up-regulation of OC gene expression in BMMSCs was observed when treated with un-depleted DDM, biglycan-depleted DDM and decorin-depleted-DDM, compared to un-treated cells. However, statistical significance was only achieved for cells cultured with decorin-depleted DDM, compared to un-treated cells. Error bars represent  $\pm$ SD (n=3). \* = P<0.05, \*\* = P<0.01.

# 5.3.8 Alizarin Red Staining of BMMSCs Cultured with Individual Immuno-Precipitated DDM Fractions

After 28 days, Alizarin red staining of BMMSCs cultured with un-depleted DDM and decorin-depleted DDM yielded visible nodules of mineralisation (white arrows). Sparse, however, dense nodule formation occured with cells cultured with biglycan-enriched DDM (black arrow). No prominent nodules of staining are present for cells cultured with biglycan-depleted or decorin-enriched DDM, akin to un-treated cells (Figure 5.8)



DEC-depleted DDM

DEC-enriched DDM

CTRL

Figure 5.8 - Micrograph images of BMMSCs stained by Alizarin red for mineral deposition analysis after 28 days in culture, with individually immuno-depleted DDM. BMMSCs cultured with un-depleted and decorin-depleted DDM produced mineralised foci (white arrows). Meagre staining was apparent for cells cultured with biglycan-depleted or decorinenriched DDM. White bars represent 100µm.

### 5.3.9 Quantification of Alizarin Red Staining of BMMSCs Cultured with Individual Immuno-Precipitated DDM Fractions

Alizarin red staining was greater for BMMSCs treated with un-depleted DDM, compared to cells treated with biglycan-depleted DDM (P<0.01), decorin-enriched DDM (P<0.01) and un-treated cells (P<0.01). Staining for cells treated with decorin-depleted DDM was unchanged from cells treated with un-depleted DDM, however, staining was greater compared with un-treated cells (P<0.01). Staining for cells treated DDM, however, staining was greater compared and decorin-enriched DDM was unchanged, compared to un-treated cells (Figure 5.9).



Figure 5.9 - Graph representing intensity of Alizarin red S staining of BMMSCs cultured with individual immuno-depleted DDM fractions for 28 days. Un-depleted and decorin-depleted DDM fractions elicited the highest levels of staining of BMMSCs. Staining was unchanged in cells cultured with biglycan-depleted DDM or decorin-enriched DDM, compared to untreated cells. Error bars represent  $\pm$ SD (n=3). \*\* = P<0.01.

# 5.3.10 Alizarin Red Staining of BMMSCs Cultured with Dual Immuno-Precipitated DDM Fractions

After 28 days, Alizarin red staining of BMMSCs cultured with un-depleted DDM yielded visible nodules of mineralisation (white arrow). Very small nodules were present for cells treated with biglycan/derocin-depleted DDM and biglycan/decorin-enriched DDM (black arrows). No staining was observed for BMMSCs cultured with basal media (Figure 5.10).

Un-depleted DDM





BGN/DEC-enriched DDM



Figure 5.10 - Micrograph images of BMMSCs stained by Alizarin red for mineral deposition analysis after 28 days in culture with dual immuno-depleted DDM. BMMSCs cultured with un-depleted DDM produced defined mineralised foci (white arrow), however, smaller deposits are present for cells cultured with biglycan/decorin-depleted DDM and biglycan/decorin-enriched DDM (black arrows). Un-treated cells did not visibly stain. White bars represent 100µm.
# 5.3.11 Quantification of Alizarin Red Staining of BMMSCs Cultured with Dual Immuno-Precipitated DDM Fractions

Alizarin red staining was greater for BMMSCs cultured with un-depleted DDM compared to cells cultured with biglycan/decorin-depleted DDM (P<0.01), biglycan/decorin-enriched DDM (P<0.05) and un-treated cells (P<0.01). There was no significant difference in staining for cells cultured with biglycan/decorin-depleted DDM or biglycan/decorin-enriched DDM compared, with un-treated cells (Figure 5.11).



Figure 5.11 - Graph representing intensity of Alizarin red staining of BMMSCs cultured with dual immuno-depleted DDM fractions, for 28 days. Un-depleted DDM provoked the highest levels of staining, however, staining was diminished when cells were treated with biglycan/decorin-depleted DDM and biglycan/decorin-enriched DDM. Error bars represent  $\pm$ SD (n=3). \* = P<0.05, \*\* = P<0.01.

#### 5.4 Discussion

The results presented within this Chapter have acknowledged the roles for biglycan and decorin in DDM for orchestrating osteogenic responses of BMMSCs. Both individual and dual depletion of biglycan and decorin resulted in the co-depletion of TGF- $\beta_1$ . Depletion of biglycan from DDM impeded osteogenic potency towards BMMSCs, accompanied with abolished mineralisation, which is not recovered with isolated biglycan. Conversely, decorin depletion from DDM has no effect on the osteogenic effects towards BMMSCs. Dual-depleted DDM resulted in the abolishment of osteogenic potential of DDM towards BMMSCs, which was not recovered with the application of isolated biglycan/decorin. Collectively, biglycan and decorin, along with TGF- $\beta_1$ , are proposed to possess differential roles in orchestrating cellular responses to DDM, which strengthens the evidence for their role in the regulation of osteogenic differentiation.

The work within this study identified that DDM contained both biglycan and decorin, in accordance with previous literature reports (Waddington *et al*, 2003b; Goldberg *et al*, 2005). For both SLRPs analysed in this study, co-depletion of TGF- $\beta_1$  was noted in both individual and dual immuno-precipitated DDM, therefore indicating interactions of both of these SLRPs with TGF- $\beta_1$ . Both biglycan and decorin have been recognised to be able to form complexes with TGF- $\beta_1$ , which can be increased by de-glycosylation of both SLRPs, thus indicating binding of TGF- $\beta_1$  is to the respective core protein component (Hildebrand *et al*, 1994). Baker *et al* (2009) reported that depletion of biglycan or decorin individually from DDM does not affect the affinity of TGF- $\beta_1$  to the matrix. However, dual depletion reduces affinity by around 50%, compared to un-depleted matrix, thus proposing roles for biglycan and decorin in sequestering TGF- $\beta_1$  within DDM.

Compared to un-depleted DDM, culturing of BMMSCs with decorin-enriched DDM resulted in enhanced cell expansion, cumulative with reduced RunX2 and OC mRNA expression; and abolished mineralisation in culture, compared to cells treated with un-depleted DDM. Conversely, the depletion of decorin from DDM did not alter RunX2 nor OC expression, or calcium accumulation, compared to un-depleted DDM. Decorin is known to possess differential proliferative effects on a variety of cells, by

reducing proliferation of fibroblasts (Zhang et al, 2007a; Ferdous et al, 2010); and enhancing proliferation of myoblasts (Kishioka et al, 2008). Consequently, controversy exists over the mechanisms of action that decorin possesses in altering cellular behaviours. As determined in this study, decorin forms a complex with TGF- $\beta_1$ , which has been proposed to lead to inactivation of the growth factor (Border *et* al, 1992), potentially by sequestering TGF- $\beta_1$  to collagenous components of the ECM (Markmann *et al*, 2000). Conversely, formation of a decorin-TGF- $\beta_1$  complex is noted to activate the growth factor and enhance bioactivity towards MC3T3-E1 preosteoblast cells (Takeuchi et al, 1994). Taken together, the formation of complexes of TGF- $\beta_1$  with decorin may be enhancing bioactivity of TGF- $\beta_1$ , and thus stimulating expansion of BMMSCs via TGF- $\beta_1$  signalling. Regarding differentiation, the results from this Chapter suggest that decorin depletion from DDM does not affect the potential for osteogenic differentiation of BMMSCs or the subsequent calcium accumulation. Intestinal epithelial cells of decorin-KO mice experience 1.6-fold greater  $\beta$ -catenin signalling, compared to wild-type cells (Bi *et al*, 2008). Additionally, decorin antagonises β-catenin levels from non-canonical Wnt signalling (Buraschi et al, 2010). Murine BMMSCs deposit an ECM containing decorin amongst other components, such as collagens I, III and V, fibronectin and biglycan, which promotes increased colony-forming efficiency and represses spontaneous differentiation (Chen et al, 2007). In vivo KO of decorin from mice has been demonstrated not to alter skeletal mass, compared to wild-type animals (Corsi et al, 2002). Concomitant with the results of this Chapter, this data suggests that isolated decorin may be acting as a repressor of osteogenic differentiation and may be redundant for formation of bone mass.

In contrast to decorin, the depletion of biglycan from DDM did not alter BMMSC expansion, however, resulted in reduced RunX2 mRNA expression and abolished potential for mineralised matrix deposition, compared to un-depleted DDM. Biglycan has been implicated in several studies in the modulation of osteogenesis. Biglycan has been shown to enhance BMP-4-induced differentiation of mouse osteoblasts, via RunX2 signalling (Chen *et al*, 2004). In rat mandible defects, BMP-2 signalling is enhanced when combined with de-glycated biglycan, compared to

biglycan associated with GAGs (Miguez *et al*, 2011). Aside from modulation of growth factor binding, biglycan is also implicated in the alteration of cell signalling and can modulate the Wnt pathway by co-binding of Wnt3a ligand and its receptor LRP6, resulting in downstream RunX2 transcription (Berensden *et al*, 2011). Interestingly, BMMSCs cultured with biglycan-depleted DDM expressed comparable mRNA levels of OC as cells treated with un-depleted DDM, despite significantly lower RunX2 expression at day 5. A possible explanation for this observation may be that although biglycan enhances expression of DDM-induced RunX2 expression, biglycan-depleted DDM could be inducing OC expression via a RunX2-independent pathway, as BMP-2 can induce expression of osterix via Dlx5, independently of RunX2 expression (Lee *et al*, 2003; Ulsamer *et al*, 2008).

Analysis of the gene expression levels for OC determined that there was no difference between expression levels in cells treated with un-depleted DDM, compared with biglycan-depleted DDM. Despite this, mineral accumulation by cells treated with biglycan-depleted DDM was abolished, compared to cells treated with un-depleted DDM. Therefore, this suggests that biglycan is indispensable for mineralisation of BMMSCs treated with DDM. In vivo, biglycan-KO mice experience reduced bone growth and skeletal mass, compared to wild-type animals (Xu et al, 1998). In addition, biglycan is highly expressed in the calluses of mouse fracture sites, with KO of biglycan resulting in reduced callus size and less woven bone (Berensden et al, 2014). In vitro, transfection of MC3T3-E1 cells with biglycan enhances matrix mineralisation, however, mineralisation is depressed in biglycan-KO cells (Parisuthiman et al, 2005). Soluble biglycan has been shown to function as a nucleator of hydroxyapatite (Gafni et al, 1999), in addition to being proposed to possess a stronger affinity for hydroxyapatite crystals than decorin, with affinity enhanced upon removal of GAG chains from the core protein (Sugars et al, 2003). Collectively biglycan possesses a key role in the mediation of matrix mineralisation.

The depletion of biglycan and decorin individually from DDM indicates differential roles for these SLRPs in the osteogenic potential of DDM towards BMMSCs. Dual depletion of these SLRPs from DDM was assessed to determine the potential for coordinated activities in these proteins for directing osteogenesis. The dual depletion of biglycan/decorin from DDM did not result in differences in BMMSC expansion, compared with un-depleted matrix. Interestingly expansion of BMMSCs with biglycan/decorin-enriched DDM was not changed compared to un-treated cells. In contrast, an increase in expansion for cells treated with biglycan-enriched and decorin-enriched DDM was noted, compared to un-treated cells. As both biglycan and decorin were demonstrated to form complexes with TGF- $\beta_1$ , their dual application to BMMSCs may have been beginning to induce a switch in fate between expansion and apoptosis, due to over-activation of TGF- $\beta_1$  signalling (Bi *et* al, 2005). Although cell expansion was not changed, compared with un-depleted DDM, the dual depletion did result in a very slight reduction of RunX2 mRNA expression, compared to un-depleted DDM. This expression was not recovered following treatment of cells with biglycan/decorin-enriched DDM. Furthermore, dual depletion of biglycan/decorin resulted in abolished mineral accumulation of BMMSCs, compared to cells cultured with un-depleted DDM. In vivo, dual KO of both biglycan and decorin render mice severely osteopenic at 2 months of age, however, only mild osteopenia is observed for mice devoid of only biglycan (Xu et al, 1998). Concurrent with the results in this Thesis, it has been reported that dual KO of biglycan and decorin from mouse BMMSCs eliminates the mineralisation effect of exogenous BMP-2, however, RunX2 expression is not affected, compared to wild-type cells (Bi et al, 2005).

In dentine, both biglycan and decorin are implicated to be profoundly involved with the process of mineralisation. Disruption of either SLRP in mice results in hypomineralised dentine, with a more marked effect observed for decorin depletion (Goldberg *et al*, 2005). Reports indicate that decorin is localised predominantly in pre-dentine along the front of mineralisation (Yoshiba *et al*, 1996), whereas biglycan is distributed uniformly throughout dentine (Orsini *et al*, 2007). Decorin is considered as a regulator of collagen fibrillogenesis by arranging collagen molecules within microfibrils and preventing lateral fusion between molecules (Weber *et al*, 1996). On the contrary, biglycan is reported to possess no influence for the promotion of fibrillogenesis (Sugars *et al*, 2003). The binding of decorin to collagen fibrils at the gap zone is additionally believed to block the initiation of mineralisation (Hoshi *et al*, 1999). Consequently, decorin is postulated to regulate mineralisation. On the other hand, biglycan is recognised to possess a predominant role in the promotion of mineralisation, as biglycan possesses a higher affinity for hydroxyapatite than decorin (Boskey *et al*, 1997); and also competes with decorin for binding sites of type I collagen (Schönherr *et al*, 1995). Furthermore, in the development of bone tissue, biglycan demonstrates biphasic manifestation with DS-conjugated, biglycan present during cell proliferation and CS-conjugated, biglycan present at the onset of mineralisation. Conversely, decorin manifestation remains constant throughout the process of bone formation (Waddington *et al*, 2003a). SLRPs present in mineralised dentine are associated with chondroitin sulphate GAG chains (Waddington *et al*, 2003b), where these conjugated GAGs are proposed to assist in mineralisation by possessing binding affinity to hydroxyapatite (Embery *et al*, 1998). Collectively, the SLRPs, biglycan and decorin, act temporally to coordinate the process of mineralisation and thus, their dual absence results in diminished potential for mineralised tissue development.

Overall, this Chapter has identified that the SLRPs, biglycan and decorin, present within DDM and possess differential roles in directing the osteogenesis of BMMSCs in response to DDM. Decorin is an expendable component of DDM for directing osteogenesis of BMMSCs *in vitro* and may be responsible for maintenance of multipotency of BMMSCs. However, *in vivo* effects in humans are not known. Conversely, biglycan is indispensable for the osteogenic potency of DDM towards BMMSCs and is required for the formation of a mineralised matrix by treated cells. Dual depletion of these SLRPs from DDM also diminishes their mineralisation potential. As a whole, SLRPs are substantially important constituents of DDM for directing repair of mineralised tissues. Chapter 6 - Assessment of Silk-Fibroin/Gelatin (SF/G) Substrates Loaded with Demineralised Dentine Matrix (DDM) for Potential in Directing Osteogenesis of Bone Marrow-Derived, Mesenchymal Stem Cells (BMMSCs)

#### 6.1 Introduction

Over recent years, the field of bone tissue engineering has expanded rapidly, with the aim of providing successful interventions for compromised bone repair, such as in cases of excessive trauma, ageing and disease-associated complications, where autologous bone grafts (ABGs) have met with numerous shortcomings (Rogers and Greene, 2012; Black *et al*, 2015). Although increasing progress is being achieved with regards to efficacious interventions for bone repair, tissue engineering of bone is currently faced with numerous challenges. Poor vascularisation of tissue-engineered grafts reduces nutrient and oxygen permeation; and subsequently results in cellular necrosis and graft failure (Kanczler and Oreffo *et al*, 2008; Rouwkema *et al*, 2008).

Growth factors, in particular the bone morphogenetic proteins (BMPs), BMP-2 and BMP-7, have been shown to demonstrate mediocre effects on *in vivo* bone regeneration in clinical trials (Friedlaender *et al*, 2001; Calori *et al*, 2008; Katayama *et al*, 2009). However, supraphysiological doses of these factors are required for clinical effect, due to their rapid proteolysis and uncontrolled diffusion (Krishnamurthy and Manning, 2002; Lee *et al*, 2011). Thus, a scaffold-based carrier is required to alleviate these issues, in addition to preventing undesirable side effects and enhancing cost effectiveness (Seeherman and Wozney, 2005; Dahabreh *et al*, 2009; Vo *et al*, 2012; Carragee *et al*, 2013).

Scaffolds used in bone tissue engineering (BTE) are required to possess numerous characteristics, to maximise the potential for successful tissue engineering of bone. Aside from osteoinductive and osteoconductive capability, other desirable properties include biocompatibility, low immunogenicity, optimal porosity, mechanical robustness and degradability (Olszta *et al*, 2007; Murphy *et al*, 2010; Bose *et al*, 2012).

Broadly, the two general classes of bone scaffolds are natural or synthetic, each with their own advantages and disadvantages. Aside from ABGs, naturally-derived, bone grafts include allografts and xenografts. Naturally-derived, bone grafts possess osteoinductive and osteoconductive properties, due to their structural similarities to natural bone and the presence of bioactive factors embedded within them (Roberts and Rosenbaum, 2012). However, due to the associated risks with disease transmission and immunoreactions, osteoinductivity of these grafts are diminished, following irradiation and freeze drying methods (Lord et al, 1988; Delloye et al, 2007; Brydone et al, 2010). Naturally-derived, collagen scaffolds are biocompatible; and promote cell adhesion and proliferation (Hiraoka et al, 2003), however, suffer from poor mechanical integrity (Harley et al, 2007). Synthetic scaffolds can be composed of various commercially available materials, including ceramics, bioactive glasses and metals. Although synthetically-derived, scaffolds are less associated with immunoreactivity; and possess osteoinductive and osteoconductive properties, they are associated with poor degradation characteristics in vivo (Rezwan et al, 2006; Staiger et al, 2006, Amini et al, 2012; Oryan et al, 2014).

Of late, silk biomaterials are receiving much attention for biomedical applications owing to desirable properties, including biocompatibility (Yang *et al*, 2007b; Shen *et al*, 2014), controllable biodegradation (Horan *et al*, 2005; Lu *et al*, 2011); and excellent mechanical properties (reviewed in Koh *et al*, 2015). Notably, silk-fibroin (SF) additionally possesses high oxygen and water permeability, in addition to eliciting minimal inflammatory reactions *in vivo* (Unger *et al*, 2007). Resultantly, numerous studies have investigated the potential for SF-based, biomaterials for use in BTE (Jones *et al*, 2009; Uchida *et al*, 2014). Incorporation of BMP-2 into silk-microparticles enhances *in vitro* and *in vivo* bone formation, compared to soluble BMP-2 (Bessa *et al*, 2010). Additionally, incorporation of BMP-2 and vascular epidermal growth factor (VEGF) into silk hydrogels has demonstrated increased *in vivo* bone formation, compared to un-loaded constructs (Zhang *et al*, 2011b).

Although silk has widely been investigated as a natural polymer for use in biomedical applications, silk-fibroin obtained from *Bombyx mori* (*B. mori*) lacks the

integrin recognition arginine-glycine-aspartic acid (RGD) and thus, experiences attenuated cell attachment properties (Minoura *et al*, 1995). Consequently, blending of additional constituents with silk-fibroin is required to achieve cell attachment. Gelatin is naturally derived from denatured collagen and contains unmasked RGD motifs (Davis, 1992; Gullberg *et al*, 1992), which confers excellent cell attachment and proliferation properties of gelatin (Dubruel *et al*, 2007; Seo *et al*, 2009; Wu *et al*, 2011). Despite its excellent cell adhesion properties, gelatin is seldom utilised alone as it is highly soluble in aqueous environments and undergoes uncontrollable degradation (Mandal *et al*, 2009). Degradation of gelatin, however, can be utilised to permit the controlled release of bioactive factors for enhancement of wound repair sites (Yamamoto *et al*, 2001; Hori *et al*, 2007).

This Chapter aimed to analyse the potential for silk-fibroin/gelatin (SF/G)-based matrices, to support the culture of bone marrow-derived, mesenchymal stem cells (BMMSCs) and to test the potential for osteogenic induction by incorporation of demineralised dentine matrix (DDM), with the intention of enhancing bioactivity of SF-based biomaterials.

# 6.2 Materials and Methods

#### 6.2.1 Processing of Silk-Fibroin (SF) and Gelatin

Dr. Paul DeBank and Kim Luetchford performed processing and casting of SF/G and SF/G/DDM constructs, in collaboration with the University of Bath.

SF was purified from raw silk of *B. mori* silk cocoons (Homecrafts Direct, UK). Silk cocoons were boiled in 0.2M sodium carbonate (Sigma-Aldrich, UK) for 1 h. Silk was washed five times in deionised distilled H<sub>2</sub>O (ddH<sub>2</sub>O) and air dried under ambient conditions. Dried silk was dissolved in 9M lithium bromide (Sigma-Aldrich) at 15% w/v, at 60°C for 4 to 5 h. The solution was cooled and dialysed against regularly changed ddH<sub>2</sub>O for 2 days. The concentration of the SF solution was determined by drying a known volume of the solution. SF solution was stored at 4°C, or lyophilised. The solution was used at a concentration of 5% w/v, unless stated otherwise.

For blending with SF, type I porcine gelatin (Sigma-aldrich) was dissolved in  $ddH_2O$  at approximately 60°C, at a concentration of 5% w/v.

### 6.2.2 Casting of 2D films of SF/G and SF/G/DDM

Aqueous solutions of SF (5% w/v) and gelatin (5% w/v) were blended in ratios of SF:G at 75:25, 50:50 and 25:75. Solutions were applied to 96-well and 12-well cell culture plates (Sarstedt, UK) at  $105\mu$ L/cm<sup>2</sup>; and were allowed to fully evaporate under ambient conditions.

For DDM inclusion into films, lyophilised DDM (obtained as described in Section 3.2.1.2) was reconstituted in the SF component of the solution and then blended with additional SF and gelatin to produce concentrations of DDM at 20, 10 and 5  $\mu$ g/mL. SF/G/DDM films were cast into tissue culture plates, as described above.

Dried films were treated with 100% (v/v) methanol (Fisher Scientific, UK) overnight to induce the conformational change of SF to insoluble  $\beta$ -sheet structure. Methanol was aspirated and the plates air-dried under ambient conditions. Dried plates were sterilised by exposure to UV light (253.7 nm), for 30 min.

#### 6.2.3 Morphological Determination of BMMSCs on SF/G and SF/G/DDM films

BMMSCs (15.2-16.8 population doublings, PDs) were seeded onto SF/G and SF/G/DDM films in triplicate 12-well plates at 4x10<sup>3</sup>cells/cm<sup>2</sup>; and cultured for 48 h. Cells were photographed under light microscopy using a Panasonic DMC-G1 Camera. 5 random and independent images were recorded per well.

#### 6.2.4 BMMSC Expansion on SF/G and SF/G/DDM Films

Expansion of BMMSCs on SF/G and SF/G/DDM films was determined using a modified protocol of the previously described thiazolyl blue tetrazolium blue (MTT) assay (Section 3.2.2). Briefly, BMMSCs (14-17.5 PDs) were seeded onto SF/G and SF/G/DDM blended films in triplicate wells of a 96-well plate. Cells seeded into wells containing no SF/G films (SF/G plates) or un-loaded SF/G films (SF/G/DDM plates) served as controls. After 0, 24, 48 and 72 h in culture, 20µL of culture medium was aspirated and replaced with 20 µL of sterile filtered MTT solution (5mg/mL in PBS) (Sigma-Aldrich). Plates were incubated and processed, as described previously (Section 3.2.1). Experiments were performed in triplicate.

The SF/G blend ratio that best supported BMMSC expansion was chosen to assess the effects of DDM inclusion on the osteogenic effects of BMMSCs.

#### 6.2.5 Osteogenic Induction Ability of SF/G/DDM Films

Osteogenic gene expression of BMMSCs over 5 days cultured on SF/G/DDM films, was performed as described previously (Section 3.2.6). Briefly, BMMSCs (17-17.5 PDs) were seeded onto SF/G/DDM films in wells of a 6-well plate (Sarstedt) at 10<sup>4</sup> cells/cm<sup>2</sup>, in un-conditioned media. Media changes were performed every 2 days. RNA was extracted using an RNeasy Mini Kit (Qiagen, Germany) (Section 2.2.3), converted to cDNA (Section 2.2.4) and quantitative real time-polymerase chain reaction (q-PCR) analysis performed for RunX2 gene expression (Section 3.2.6). GAPDH was used as an internal reference for normalisation of data. Primer sequences are detailed in Table 6.1. Experiments were performed in triplicate.

Gene	Primer sequence: 5'-3'	Annealing	Product	Primer
product		temperature	length	designer
		(°C)	(bp)	
GAPDH	F: TTCTTTTGCGTCGCCAGCCGA	55	96	Author
	R: GTGACCAGGCGCCCAATACGA			
RunX2	F: GGTTAATCTCCGCAGGTCACT	55	80	Author
	R: CCCTCTGTTGTAAATACTGCTTGC			

Table 6.1 - Details of primer sequences used for q-PCR analysis of genes expressed by BMMSCs cultured on SF/G films.

# 6.2.6 Mineral Deposition Analysis of BMMSCs on SF/G/DDM Films

Mineral deposition of BMMSCs cultured on SF/G/DDM films and subsequent Alizarin red staining was performed, as described previously (Sections 3.2.5.1-3.2.5.2). Briefly, BMMSCs (17-17.5 PDs) were seeded onto SF/G/DDM films in 12-well plates at  $10^4$  cells/cm<sup>2</sup> in un-conditioned media. Media changes performed every 2 days. After 28 days in culture, media was aspirated, cells washed with phosphate buffered saline (PBS), fixed with 10% (v/v) formaldehyde (Sigma-Aldrich); and stained with 2% Alizarin red (Sigma-Aldrich). Stained cells were washed with ddH<sub>2</sub>O, air-dried and photographed under light microscopy using a Panasonic DMC-G1 camera. Experiments were performed in triplicate.

#### **Statistical Analyses**

Statistical analyses were performed utilising One-Way ANOVA with a post-hoc Tukey test using GraphPad InStat 3 (v3.06). Statistical values were defined as significant (\*, P<0.05), very significant (\*\*, P<0.01) or extremely significant (\*\*\*, P<0.001).

# 6.3 Results

### 6.3.1 BMMSC Morphology on SF/G Blended Films

BMMSCs seeded onto substrate blends of SF/G demonstrate cellular attachment to all blend ratios tested. BMMSCs seeded onto 75SF/25G and 50SF/50G blend exhibit fibroblast-like morphology, similar to cells cultured on plastic. BMMSCs on the 25SF/75G blend, however, demonstrate appearance of associated globular structures and appear smaller (black arrows) (Figure 6.1).



75SF/25G

Plastic

Figure 6.1 - Morphological appearance of BMMSCs on substrate blends of SF/G, 48 h postseeding. BMMSCs demonstrate fibroblast-like morphology on 50SF/50G and 75SF/50G blend ratios, however, morphology of cells cultured on 25SF/25G substrate appears altered. White bars represent 100µM.

#### 6.3.2 BMMSC Expansion on SF/G Blended Films

SF/G substrate blends provided a surface that facilitated expansion of BMMSCs over 72 h. Higher ratios of SF within the substrate blends appeared to support cell expansion to the greatest degree, with SF at 50% and 25% causing a significant reduction in cell expansion after 48 h, compared to BMMSCs cultured on plastic. After 72 h in culture, expansion of cells on 75SF/25G was not different compared to cells cultured on plastic (Figure 6.2).



Figure 6.2 - Expansion of BMMSCs on SF/G substrate blends. Expansion of BMMSCs cultured on SF/G at a blend ratio of 75:25 was not changed, compared to cells cultured on plastic, after 72 h. Error bars represent  $\pm$ SD (n=3). \* = P<0.05, \*\*=p<0.01, \*\*\*=p<0.001.

# 6.3.3 BMMSC Morphology on SF/G/DDM Films

BMMSCs seeded onto substrate blends of SF/G demonstrate cellular attachment to all blend ratios tested in addition to fibroblast-like morphology (Figure 6.3), akin to BMMSCs seeded onto plastic (Figure 6.1).



5µg/mL DDM

0µg/mL DDM

Figure 6.3 - Morphological appearance of BMMSCs on 75% SF/25% gelatin, loaded with DDM at concentrations of 0-20 $\mu$ g/mL, 48 h post-seeding. BMMSCs demonstrate fibroblast-like morphology on substrates at all DDM concentrations tested. White bars represent 100 $\mu$ M.

#### 6.3.4 BMMSC expansion on SF/G/DDM Films

DDM-loaded SF/G substrates supported cell expansion over 72 h. After 24 h, BMMSCs cultured on SF/G loaded with DDM at  $10\mu$ g/mL underwent significantly greater expansion, compared to cells cultured on un-loaded SF/G substrates (P<0.05). After 72 h, BMMSCs cultured on SF/G loaded with DDM at  $20\mu$ g/mL had expanded significantly more compared to cells cultured on un-loaded SF/G (P<0.05) (Figure 6.4).



Figure 6.4 - Expansion of BMMSCs on DDM-loaded SF/G substrates. Loading of DDM at  $20\mu g/mL$  enhanced BMMSC expansion, the greatest compared to un-loaded constructs after 72 h. Error bars represent ±SD (n=3). \* = P<0.05.

#### 6.3.5 RunX2 Gene Expression of BMMSCs on SF/G/DDM Films

RunX2 expression of BMMSCs cultured on SF/G films was not changed after 5 days in culture; and was comparable to the expression level of RunX2 in cells cultured on plastic. No differences in RunX2 expression level between BMMSCs cultured on SF/G films loaded with varying DDM concentrations were observed (Figure 6.5).



Figure 6.5 – RunX2 expression of BMMSCs cultured on DDM-loaded SF/G substrates after 5 days. No differences for RunX2 expression were observed during the culture period. Error bars represent  $\pm$ SD (n=3).

# 6.3.6 Mineral Deposition Analysis of BMMSCs Cultured on SF/G/DDM Films

Alizarin red staining of BMMSCs cultured on SF/G substrates did not reveal any distinct nodules. However, faint staining is visible for cells cultured on SF/G substrates loaded with the various concentrations of DDM (black arrows). No staining was visible for BMMSCs cultured on un-loaded SF/G (Figure 6.6).



5µg/mL DDM

0µg/mL DDM

Figure 6.6 - Micrograph images for BMMSCs cultured on SF/G substrates stained with Alizarin red after 28 days in culture. No prominent mineralised nodules were apparent for any group tested, however, faint staining is present for cells cultured in substrates loaded with DDM. White bars represent 100µM.

#### 6.4 Discussion

Preliminary data obtained in this Chapter demonstrated that SF/G blended films supported expansion of BMMSCs, which was enhanced by additional blending with DDM, thus indicating the successful incorporation of DDM into SF/G films. Furthermore, osteogenic potential of DDM-loaded SF/G films was demonstrated, indicating their potential as a substrate carrier for DDM.

Numerous methods have been developed to prepare SF based constructs, including electrospinning (Park *et al*, 2010; Liu *et al*, 2013), freeze drying (Zhang *et al*, 2010; Mandal *et al*, 2013); and salt-leaching (Marolt *et al*, 2006; Bhumiratana *et al*, 2011). Methods in this Thesis utilised the generation of SF/G films via casting, which has also been demonstrated as a preparation method for silk-based biomaterials (Mieszawska *et al*, 2010; Yao *et al*, 2012). This method was chosen so that direct comparisons could be made between BMMSCs seeded onto SF/G constructs and on to tissue culture plastic. SF films also demonstrate good mechanical strength when wet, in addition to excellent permeability of small molecules (Yang *et al*, 2015).

BMMSCs seeded onto SF/G films at respective blend ratios of 75:25, 50:50 and 25:75 all demonstrated cell attachment coupled with fibroblast-like morphology of cells, comparable to culture on tissue culture plastic. Blending of SF and gelatin has previously been demonstrated to generate constructs that have successfully promote adhesion of hepatic (Yang et al, 2012) and fibroblastic (Yang et al, 2015) cell lines. Regarding cellular expansion, after 72 h in culture, a trend of a decrease in cell expansion was observed with increasing gelatin concentration, compared to cells cultured on plastic. Film constructs consisting of pure SF have demonstrated viability of human MG63 osteoblast-like cells equivalent to that of cells cultured on tissue culture surfaces, thereby indicating very high biocompatibility (Varkey et al, 2015; Yoo et al, 2016). Blending of gelatin at concentrations of 10-20% with SF has been shown to provide superior proliferation of fibroblasts, compared to pure gelatin; and was postulated that SF can enhance the mechanical integrity of gelatin films to provide an optimal surface to permit cell growth (Yang et al, 2015). Concurrent with Yang et al (2015), the SF/G blend of 75SF/25G in this Thesis constituted a substrate for permitting expansion of BMMSCs. Therefore, this blend

ratio was further tested to analyse the effects of DDM-incorporation into SF/G films for directing osteogenesis of BMMSCs.

Incorporation of DDM into SF/G films in this Thesis did not alter BMMSC morphology, however, loading of SF/G films with 20µg/mL of DDM resulted in significant enhancement of BMMSC expansion after 72 h, compared to un-loaded SF/G films. Studies analysing loading of SF based matrices with bioactive factors have additionally demonstrated positive results regarding modulating cellular behaviours. Incorporation of VEGF in SF/calcium phosphate enhances proliferation of human osteoblasts, compared to un-loaded constructs (Farokhi *et al*, 2014). Also, addition of transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) to SF/chitosan 3D scaffolds increases cell adhesion and proliferative activity of human osteoblasts (Tong *et al*, 2016). The presence of identified growth factors in DDM (Sections 4.3.2 and 4.3.3) that are reported to encourage BMMSC expansion, such as TGF- $\beta_1$  and fibroblast growth factor-2 (FGF-2), suggests that these factors have been successfully incorporated into the SF/G substrates and are being released and/or presented to cells.

To further determine the bioactive potential of SF/G/DDM films, osteogenic induction ability was investigated. After 5 days in culture, culture of BMMSCs on un-loaded SF/G films did not promote a detectable change in RunX2 gene expression compared with cells cultured on tissue culture plastic. Incorporation of DDM at concentrations of up to 20µg/mL of scaffold additionally did not detect a change in RunX2 expression of BMMSCs after 5 days. However, Alizarin red staining was used as a further measure to identify the osteogenic potential of SF/G constructs in longer-term culture. Whilst no staining for mineralisation was evident for cells cultured on un-loaded SF/G films, staining was evident for SF/G films, although a lack of observed change in RunX2 gene expression after 5 days, may be as a result of slow release of DDM from the scaffold. Therefore, any increased expression may have been delayed and not detected in at the time point tested. The identification of mineralised nodules, however, suggests that loading of SF/G with DDM may constitute a mildly osteoinductive matrix for BMMSCs

Whilst research into the bone forming potential of un-loaded SF constructs has yielded varying results, additional articles have investigated the potential of SF constructs loaded with bioactive factors to promote an appropriate fabrication for enhancement of bone repair. Encapsulation of BMP-2 into SF micro-particles increases mineralisation of C2C12 cells, compared to treatment of soluble BMP-2 *in vitro*, in addition to enhancing bone formation in a rat ectopic model 2 weeks after implantation *in vivo* (Bessa *et al*, 2010). Incorporation of BMP-2 and VEGF into SF hydrogels additively augment bone repair in rabbit sinus floor elevation surgeries 12-weeks post implantation, whereas un-loaded SF hydrogels did not promote sufficient bone regeneration (Zhang *et al*, 2011b). The use of gelatin as a delivery vehicle for growth factors in bone repair has also been investigated by Furuya *et al* (2015), who reported that loading of gelatin hydrogels with FGF-2 enhances repair of bone in murine femur fractures, compared to FGF-2 free in solution. Currently, studies investigating the efficacy of growth factor loading in SF/G composite constructs are scarce.

In this Thesis, the chosen blend ratio of 75SF/25G, although permitted the highest level of cell expansion, may not be suitable for promoting optimal release of entrapped bioactive factors. The reduced cell expansion observed for SF/G films with a greater proportion of gelatin may be related to a switch in fate between cell expansion and differentiation (Stein and Lian, 1993). A major consideration of the design of supportive matrices for tissue engineering is the rate of degradation. The rate of degradation of SF-based matrices is reliant on several factors, including method of fabrication,  $\beta$ -sheet crystallinity, matrix morphology and the degrading enzymes (reviewed in You et al, 2013). Dependent on processing methods, 3D silk scaffolds implanted in vivo in sub-cutaneous pockets of rats degrade fully over periods of weeks for aqueous-cast with low  $\beta$ -sheet conformation, to over a year for constructs prepared with solvent casting and high  $\beta$ -sheet conformation (Horan et al, 2005). In vitro, treatment of SF films with methanol to induce formation of  $\beta$ sheet structure results in reduced degradation rate, compared to un-treated films (Jin et al, 2005). Furthermore, composites of SF/G have been shown to degrade more rapidly in vivo with increasing ratios of gelatin to SF (Yang et al, 2012).

Compared with the previously characterised osteogenic potential of soluble DDM in culture in this Thesis, the low osteoinductive potential for DDM-loaded SF/G films demonstrated in this Chapter may, therefore, result from insufficient release of bioactive factors and thus, resulting in hindered osteoinductive potential. Concurrent with the visually observed degradation of SF/G films composed of 25SF/75G, increasing the proportion of gelatin relative to silk-fibroin in DDM-loaded films would likely result in increased construct degradation and subsequent release of bioactive DDM. Experimentation of DDM loading to SF/G films with a higher proportion of gelatin would be beneficial in testing this hypothesis. The rate of degradation *in vitro*, however, is likely to vary largely from that of *in vivo*, as macrophages and other large, multi-nucleated cells have been reported to concentrate around SF scaffold and tissue interfaces when implanted *in vivo* (Wang *et al*, 2008).

Concentrations of factors loaded into SF based constructs are also a pivotal consideration. Although DDM at 10µg/mL has previously been demonstrated in this Thesis to induce osteogenic responses of BMMSCs, the release of biologically active concentrations of DDM from SF/G films is likely to not have been achieved. Although SF based constructs loaded with growth factors have been successful for promoting osteogenesis, the concentrations of these factors are up to the range of mg/mL of scaffold material (Li *et al*, 2006; Zhang *et al*, 2011b). Therefore, the rate of a degradation of SF/G films, in conjunction with low concentrations of loaded DDM, may be responsible for the lack of any observed osteogenic potential. Collectively, modification of SF fabrications may be necessary to induce sufficient osteogenic potential and thus, further experimentation is required to fully examine the potential of SF/G constructs for driving osteogenesis of BMMSCs.

To date, various studies have investigated the potential for SF constructs in directing bone repair *in vivo*, with varying results reported. Critical-sized calvarial defects in mice filled with porous, 3D SF scaffolds either un-loaded or loaded with MSCs, demonstrated no considerable bone formation as determined by no observed mineralisation and lack of osteocalcin (OC) expression after 5 weeks (Meinel *et al*, 2005). Conversely, almost complete bone regeneration has been

achieved by filling of rabbit calvarial defects with SF nanofibrous meshes for 12 weeks (Kim *et al*, 2005). Studies investigating the effects of gene expression of SF constructs are more limited, however, scaffolds of SF modified with hydroxyapatite induce up-regulation of gene expression of RunX2, collagen type I and osteopontin (OPN) of rabbit BMMSCs after 2 weeks, compared to unmodified SF scaffolds (Jiang *et al*, 2013). Even though mineralisation of BMMSCs cultured on SF/G/DDM films analysed in this Thesis was low, SF possesses high potential for promoting mineralisation. Whilst debated, it is postulated that the  $\beta$ -sheet structure of SF is anionic and may present nucleation sites for the deposition of hydroxyapatite (HAp) nanocrystals (Marelli *et al*,2012), or potentially that  $\beta$ -sheet crystals of SF act as sites of nucleation for HAp deposition (Vetsch *et al*, 2015). SF films have also demonstrated the ability to support extensive mineralised matrix deposition by human osteoblasts (Jin *et al*, 2015).

Overall, this Chapter has preliminarily identified that SF/G blends can support cell attachment and expansion. Loading of this SF/G blend with DDM at a range of concentrations tested provoked an osteogenic response of BMMSCs, evidenced by the identification of sparse and weakly stained nodules. Further testing of modified SF/G/DDM constructs is needed to fully determine the potential for loading of SF/G based matrices with bioactive factors for use in tissue engineering of bone.

#### Chapter 7 - General Discussion

The overall aim of this Thesis was to assess the bioactive effects of demineralised dentine matrix (DDM) for directing osteogenesis of bone marrow-derived, mesenchymal stem cells (BMMSCs) and to identify the key components of DDM that elicits these responses. It was demonstrated that DDM elicited concentrationdependent responses of BMMSCs with regards to reducing cell expansion (0.1-10µg/mL), attenuating apoptosis (10µg/mL) and encouraging cell migration (0.1µg/mL). Pertinently, DDM at 10µg/mL directed osteogenic differentiation of BMMSCs, as determined by increases in osteogenic gene expression of RunX2 and collagen type I, in addition to stimulating matrix mineralisation. Analyses of DDM identified a plethora of growth factors related to osteogenesis, including transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ), bone morphogenetic protein-2 (BMP-2) and vascular epidermal growth factor (VEGF). However, fractionation of these growth factors from DDM resulted in highly attenuated osteogenic potency, as evaluated by diminished RunX2 expression by BMMSCs, in addition to attenuated mineral deposition. Furthermore, biglycan and decorin, present with DDM, were identified to possess differential roles for directing the osteogenesis of BMMSCs; decorindepletion not affecting osteogenic potency, however, biglycan-depletion resulted in diminished osteogenic potency of DDM. Overall therefore, the matrix of DDM is considered to be highly complex and contains a multitude of constituents required for directing osteogenic differentiation of BMMSCs.

The heterogeneity of mesenchymal stem cells (MSCs) as a result in variations of tissue source, method of acquisition and *in vitro* culture protocols is well acknowledged; and is regarded as responsible for discrepancies between literature reports regarding MSC behaviours (reviewed in Wagner *et al*, 2006). In this Thesis, the isolation of fibronectin-adherent cells from a commercially available batch of BMMSCs (acquired from Lonza, UK), resulted in a more primitive population of cells with enhanced expansive capacity *in vitro*, as previously demonstrated (Dowthwaite *et al*, 2004; Waddington *et al*, 2009), thereby increasing homogeneity and providing an additional level of standardisation of the MSCs utilised in experimental procedures.

This study identified that DDM elicited a variety of effects on BMMSCs, such as reducing cell expansion, reducing apoptosis and providing a migration gradient. Most importantly, DDM stimulated the differentiation of BMMSCs towards an osteoblast lineage and resulted in matrix mineralisation. For successful bone regeneration to be achieved, there is a widely regarded consensus relating to appropriate differentiation of endogenous MSCs to sufficiently migrate to the site of injury, expand and differentiate into osteoblasts, which are responsible for the formation of reparative bone tissue (Knight and Hankenson, 2013; Wang *et al*, 2013; Açil *et al*, 2014). Collectively, the responses of BMMSCs to DDM further validate previous reports that DDM can induce reparative bone formation *in vivo* (Gomes *et al*, 2008; deOlivera *et al*, 2013).

Analyses of DDM identified the presence and elucidated the concentrations of a wide array of growth factors implicated in directing osteogenesis of mesenchymal stem cells (MSCs). Notably, TGF- $\beta_1$  and BMP-2 were present at relatively high concentrations (ng/mg), whereas other factors associated with osteogenic differentiation of MSCs such as BMP-4 and BMP-7, were identified at much lower concentrations (pg/mg). Critically, the concentrations of these factors in DDM that resulted in osteogenic responses of MSCs were much lower than concentrations of factors used in clinical practice, where concentrations of growth factors are used at µg levels. BMP-2 and BMP-7 are commercially available for clinical intervention of bone repair, however, their effectiveness is regarded as limited due to a lack of clinical trials demonstrating results for these growth factors in providing significant enhancement of clinical outcome, in addition to a caveat of cost-effectiveness, resulting from supra-physiological doses required for effect (Garrison et al, 2007; Gautschi et al, 2007). The combination of growth factors in DDM suggests that these factors may be working in co-operatively with each other, as several studies have determined that growth factor-driven, differentiation of MSCs towards an osteoblast phenotype can be synergistically enhanced using combinations two or more growth factors (Kugimiya et al, 2005; Yuan et al, 2013; Açil et al, 2014). The range of growth factors present in DDM may, therefore, be more beneficial for enhancing bone repair than single growth factors that are currently in clinical practice.

A key finding in this Thesis was that fractionation of DDM in an attempt to isolate growth factors, resulted in diminished bioactivity towards BMMSCs, compared to unfractionated matrix. Increasing reports are highlighting the interactions of growth factors with other extracellular matrix (ECM) components, which are proposed to maintain a reservoir of growth factors; and these require proteolytic activation to release these factors into the local environment for bioactivity to be conferred (Schönherr and Hausser, 2000; Wilgus, 2012). Mounting evidence is also becoming available that demonstrate the importance of the ECM in conferring bioactivity of growth factors (Martino *et al*, 2011; 2014). In this Thesis, the requirement for biglycan and decorin in the osteogenic potency of DDM was elucidated, with differential roles identified for each of these proteins. Notably, the absence of biglycan from DDM resulted in attenuated osteogenic potency towards BMMSCs, which was not rescued following treatment of cells with enriched biglycan fraction of DDM, therefore suggesting a requirement for synergistic interactions between both growth factors and matrix components to provide bioactivity.

Successful tissue engineering for bone requires the emulation of an environment that closely mimics the tissue in question to replicate the *in vivo* cell and matrix interactions; and is considered one of the biggest hurdles to overcome (Geckil *et al*, 2010). Reports are available that demonstrate growth factor efficacy can be enhanced by additional ECM constituents. Martino *et al*, (2011) demonstrated that a fibrin matrix cross-linked to fibronectin resulted in promiscuous binding of BMP-2 and platelet-derived growth factor-BB (PDGF-BB) to the fibrin matrix. When placed into a sub-cutaneous implant model, fibronectin-bound growth factors resulted in the formation of organised bone tissue, whereas negligible bone tissue was observed for growth factors that were administered, but not bound to fibronectin. Furthermore, blocking of integrin  $\alpha$ 5 $\beta$ 1 significantly reduced fibronectin-bound growth factor stat growth factors, thus suggesting that growth factor signalling may be mediated through integrin binding. A later study identified that modification of BMP-2 and PDGF-BB

with a domain from placental growth factor-2 (PIGF-2), induced exceptional affinity towards ECM proteins, such as fibronectin, fibrinogen and heparan sulphate. Interestingly, the delivery of these PIGF-2 modified growth factors resulted in markedly greater deposition of bone tissue in a rat calvarial defect model, compared to unmodified growth factors. Critically, the responses elicited for PIGF-2-modified growth factors were observed at a dose 5-fold smaller than for topically applied growth factors (Martino *et al*, 2014). Previous proteomic analyses dentine revealed the presence of fibronectin, fibronigen and heparin sulphate (Park *et al*, 2009; Jágr *et al*, 2012); and therefore may be critical ECM components required for the optimal osteogenic potential for DDM.

Incorporation of DDM in silk-fibroin/gelatin (SF/G) films resulted in a mild, yet positive, osteogenic response of BMMSCs, as determined by the onset of mineralisation. Intriguingly, mineralisation was achieved with a single dose of DDM incorporated into the films, compared to previous Chapters where repeated doses were administered. Several previous reports indicate numerous benefits for silkbased materials in bone tissue engineering (reviewed in Midha et al, 2016); and incorporation of bioactive factors have demonstrated bone forming ability in vivo, however, using concentrations of growth factors in the range of mg/mL (Zhang et al, 2011). An important consideration for loading of bioactive factors into scaffolds is the rate of release. It has been demonstrated that sustained release of BMP-2 over 4 weeks in vivo resulted in significantly greater bone formation, compared to short release over 3 days (Jeon *et al*, 2008). As such, the low concentrations of DDM incorporated in SF/G films that resulted in osteogenic responses of BMMSCs suggests entrapment of DDM within the substrate and steady, sustained release of bioactive factors that stimulated cellular responses. The degradability of SF/G matrices in vitro, however, is likely to differ largely to those that would occur in vivo, due to the presence of marcophages and other degrading cells that have been shown to concentrate around implanted silk-fibroin matrices (Wang et al, 2008).

A further critical consideration of mimicking the environment for bone tissue engineering is the promotion of vascularisation, which is required to allow the introduction of oxygen and nutrients to the centre of implanted grafts, without which graft necrosis may occur (Lascheke *et al*, 2006). At the fracture sites of bone, VEGF is regarded as the most important contributor to vascularisation (Keramaris *et al*, 2008). Whilst it has been demonstrated that human DDM can accelerate the healing of rat tooth sockets, this observation was accompanied by enhanced VEGF synthesis and blood vessel formation; and is postulated that osteogenic potential of DDM was attributable to the stimulation of angiogenesis (Reis-Filho *et al*, 2012). Vascularisation of bone defects induced by VEGF on collagen sponges has been promoted in rabbits, also suggesting that osteogenesis and angiogenesis are dependent processes (Geiger *et al*, 2005). Investigations into the potential for VEGF in DDM to promote vascularisation in bone defects *in vivo* may be beneficial for determining the extent of VEGF signalling in DDM-induced bone repair.

The potential for autologous dentine implantation for bone augmentation in implant sockets have previously been described in humans, with positive results for bone healing recognised. This healing was achieved even despite extensive processing and sterilisation procedures (Kim et al, 2010; Pang et al, 2016), however, the small volume attainable from autologous dentine restricts the potential for grafting to larger defect sites. In this Thesis, DDM pooled from different donors demonstrated positive osteogenic effects on BMMSCs from a single donor, thus suggesting DDM may be beneficial as an allogenic grafting material. Allogenic dentine has also been successfully utilised in rabbit models, to enhance bone formation in surgically induced bone defects accompanied with an apparent lack of inflammation (Bakhshalian et al, 2013). Furthermore, the potential for xenogenic dentine grafting from humans into rabbits for bone augmentation has also been investigated; and demonstrated enhanced bone formation with minor associated inflammation (Al-Asfour et al, 2013). Compositional comparisons between humanand animal-derived, dentine have determined that bovine dentine demonstrate the highest similarities compared to other species, such as porcine and ovine (Teruel et al, 2015). Collectively, the use of xenogenic dentine for investigations into the potential for bone augmentation may be an alternative option to alleviate the shortcomings of using autologous dentine. However, current research into the use of xenogenic dentine on human-derived, cells is scarce. Therefore, trials

investigating the biocompatibility of xenogenic dentine on human cells are required to determine the potential for consideration of dentine from alternative sources to be used clinically for bone repair interventions.

The bioactivity and osteogenic induction ability of dentine has been studied within previous literature reports, in addition to within this Thesis. Whilst the osteogenic potential of dentine was investigated in this Thesis, further evidence is presented for the osteogenic potential of constituents present within the enamel of teeth. The principle protein component of enamel is amelogenin, which is secreted by ameleoblasts and represents around 90% enamel proteins, amongst much less abundant proteins, such as enamelin and ameloblastin (Fincham and Simmer, 1997; Robinson et al, 1998). Amelogenin is not expressed in primary odontoblasts, however, it is present in secondary odontoblasts 4 weeks-post after the introduction of a cavity within teeth. In vitro, amelogenin is present in dental pulpderived, mesenchymal stem cells (DPSCs) induced to differentiate into odontoblasts at sites of mineralisation (Mitsiadis et al, 2014). Furthermore, exogenous application of amelogenin to BMMSCs induced to differentiate into osteoblasts amplifies mRNA expression of type I collagen, osteopontin (OPN) and bone sialoprotein (BSP), in addition to increased mineralised matrix deposition (Tanimoto et al, 2012). Consequently, additional proteins derived from dental tissues may be of interest for investigation into their osteogenic properties.

Overall, within this Thesis, it was determined that a multitude of components of DDM including both growth factors and matrix proteins are essential for orchestrating osteogenic responses of BMMSCs. However, the precise composition of components present within the vast proteome of DDM required for bioactivity is yet to be elucidated, although increasing it is becoming acknowledged that growth factor interactions with other ECM proteins are key mediators in inducing repair of bone. The use of a scaffold for DDM may also be of great benefit for aiding in release kinetics of bioactive factors for optimal effect. Whilst current research has largely focussed on the use of autologous and allogenic dentine for bone the shortcomings associated with autologous/allogenic dentine for grafting. Further

work investigating the influence of additional constituents of DDM required for osteogenesis would be beneficial for determining defined combinations of components required for optimal osteogenic potency and therefore, would assist in the development of successful tissue engineered interventions for enhanced bone repair.

#### Chapter 8 - Future work

Various efforts have been made to provide clinically available interventions to assist in the augmentation of bone, including bone grafts (Black *et al*, 2015), growth factors (Katayama *et al*, 2009), and biocompatible scaffolds (Bose *et al*, 2012; Oryan *et al*, 2014). However, currently available treatments are largely ineffective or are fought with further complications, which negate their efficacy. The results obtained and discussed within this Thesis support a growing body of evidence for the use of demineralised dentine matrix (DDM) as an alternative solution to bone grafting (Gomes *et al*, 2008; Reis-Filho *et al*, 2012; de Oliveira *et al*, 2013). As also identified, DDM constitutes a complex matrix consisting of a variety of components that elicit bioactive effect, which was shown not to be recapitulated by isolating selected components. In conjunction with existing data relating to the limited efficacy of single growth factors for augmenting bone repair clinically (Calori *et al*, 2008; Katayama *et al*, 2009), it is becoming increasingly evident that successful bone repair strategies require mimicking of complex biological matrices.

Although osteogenic gene expression and mineralisation of bone marrow-derived, mesenchymal stem cells (BMMSCs) as a result of culturing with DDM was identified in this Thesis, there is currently a lack of knowledge relating to cellular signalling events that are induced to elicit such responses. There are a wide range of intracellular signalling cascades associated with the differentiation of mesenchymal stem cells (MSCs) down the osteogenic lineage, including Smad (Song *et al*, 2010), Wnt (Gaur *et al*, 2005) p38 and extracellular regulated kinases (ERK) (Artigas *et al*, 2014). As there is a large degree of cross-talk between these pathways (reviewed in Rodríguez-Carballo *et al*, 2016), determining the extent of DDM to induce these pathways will provide further evidence for bioactivity and osteogenic potency. In particular, comparisons of pathway activations by DDM compared to bone matrix, and by individual matrix components (i.e. growth factors) would assist in determining comparative efficacy of each potential treatment, and may provide evidence to drive further research into using DDM-based treatment modalities for clinical bone repair.

Although dentine preparations have shown to provide efficacious induction of reparative bone, a caveat of limited dentine harvestable from human teeth largely restricts clinical potential to that in dental applications, accompanied with very limited translation to orthopaedic applications. As has been researched within the relevant literature, scaffolds loaded with bioactive components elicit enhanced bone repair responses compared to un-loaded matrices and individually applied growth factors (Bessa et al, 2010; Zhang et al, 2011b). As discussed in Chapter 6, incorporation of DDM into a biocompatible scaffold may maximise the potential for efficacy and could also provide a route for translating DDM-based interventions for bone repair clinically. Additional investigations into the potential for DDM loaded into silk-fibroin/gelatin (SF/G) matrices in vitro will be able to further determine the potential for stimulating biological responses to aid in the bone repair process, in addition to elucidating characteristics including degradation and release of incorporated bioactive constituents. Successful optimisation of DDM-loaded SF/G substrates, for directing bone repair responses in vitro, could lead in to preliminary in vivo investigations in rodent models to test the potential for clinically translatable DDM-based interventions for augmenting bone. The use of such a substrate carrier to deliver optimal controlled doses of biologically active signalling components could also reduce the amount of DDM required, and subsequently would encourage translation into orthopaedics, where volumes of bone required for augmentation are typically larger than those in applications in dentistry.

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