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ANALYSING THE BIOACTIVE MAKEUP OF DEMINERALISED DENTINE MATRIX ON BONE MARROW MESENCHYMAL STEM CELLS FOR ENHANCED BONE REPAIR

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Abstract

Dentine matrix has proposed roles for directing mineralised tissue repair in dentine and bone; however, the range of bioactive components in dentine and specific biological effects on bone-derived mesenchymal stem cells (MSCs) in humans are less well understood. The aims of this study were to further elucidate the biological response of MSCs to demineralised dentine matrix (DDM) in enhancing wound repair responses and ascertain key contributing components. Dentine was obtained from human teeth and DDM proteins solubilised with ethylenediaminetetraacetic acid (EDTA). Bone marrow derived MSCs were commercially obtained. Cells with a more immature phenotype were then selected by preferential fibronectin adhesion (FN-BMMSCs) for use in subsequent *in vitro* assays. DDM at 10 μg/mL reduced cell expansion, attenuated apoptosis and was the minimal concentration capable of inducing osteoblastic differentiation. Enzyme-linked immunosorbent assay (ELISA) quantification of growth factors indicated physiological levels produced the above responses; transforming growth factor β (TGF-β1) was predominant (15.6 ng/mg DDM), with relatively lower concentrations of BMP-2, FGF, VEGF and PDGF (6.2-4.7 ng/mg DDM). Fractionation of growth factors from other DDM components by heparin affinity chromatography diminished osteogenic responses. Depletion of biglycan from DDM also attenuated osteogenic potency, which was partially rescued by the isolated biglycan. Decorin depletion from DDM had no influence on osteogenic potency. Collectively, these results demonstrate the potential of DDM for the delivery of physiological levels of growth factors for bone repair processes, and substantiate a role for biglycan as an additional adjuvant for driving osteogenic pathways.

Key words: Mesenchymal stem cells, dentine, growth factors, biglycan, decorin, cell expansion, osteoblast differentiation, migration, apoptosis.

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Introduction

A plethora of growth factors, such as various isoforms of transforming growth factor β (TGF-β), bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), fibroblast growth factors (FGF), platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF) are widely implicated in directing the early events of osteogenesis (Dimitriou *et al*., 2005). These growth factors have all been identified to play crucial roles in promoting cell migration to the repair site, proliferation and differentiation of endogenous MSCs to form bone synthesising osteoblasts (Roberts and Rosenbaum, 2012), in addition to promoting angiogenesis (Geiger *et al*., 2005). Indeed, it is against background research of growth factor roles as isolated entities that BMP-2 and BMP-7, have both been approved for therapeutic use in clinical practice (Axelrad and Einhorn, 2009). However, problems exist related to toxicity of the supraphysiological doses required to evoke a stimulatory response. Resultantly, the true efficacy of clinical success is questioned due to the potential for stimulating ectopic bone formation and inflammation (Zara *et al*., 2011; Lee *et al*., 2011). These issues have partially been addressed through administering combinations of growth factors where lower dosage levels have demonstrated enhanced osteogenic bioactivity *in vitro* and *in vivo* compared to individual factors (Kanczler *et al*., 2010; Tachi *et al*., 2011; Simmons *et al*., 2004); strengthening evidence for the activities of multiple growth factors acting synergistically to deliver optimised signalling (Chen *et al*., 2010).

Dentine is recognised to possess the capacity to undergo endogenous regeneration in response to carious injury, where demineralisation results in the dissolution of bioactive constituents within the matrix. These released bioactive factors stimulate resident dental pulp-derived stem cells (DPSCs) to differentiate into odontoblast-like cells, which secrete a reparative mineralised matrix that is comparable in structure to bone (Smith *et al.*, 1995; Arana-Chavez and Massa, 2004). Due to the bioactive potential of dentine *in vivo* being limited to stimulating reparative responses within teeth, more recent studies have investigated the potential of demineralised dentine matrix (DDM) to stimulate bone repair. Placement of dentine *in vivo* has been reported to stimulate reparative bone formation in rabbit critical defects (Gomes *et al.*, 2008) and in rat tooth sockets (de Oliveira *et al.*, 2013). *In vitro*, DDM has been demonstrated to induce mineralisation and increased expression of bone forming markers of human DPSCs (Lee *et al.*, 2015; Gonçalves *et al.*, 2016; Liu *et al.*, 2016) and murine bone marrow MSCs (BMMSCs) (Yu *et al.*, 2014). Proteomic studies of human dentine have identified the presence of up to 289 different components, with TGF-β1 identified as a prominent growth factor (Jágr *et al.*, 2012; Chun *et al.*, 2011; Park *et al.*, 2009). Immuno-histochemical studies have also identified the additional presence of BMP-2 (Casagrande *et al.*, 2010), vascular epidermal growth factor (VEGF) (Roberts-Clark and Smith, 2000) and IGF (Finkelman *et al.*, 1990). Whilst the synergistic signalling activities of these growth factors are acknowledged in contributing to osteoblast differentiation and directing bone repair, there is an increasing recognition for contributions by other matrix constituents which are proposed to act to tether growth factors to the extracellular matrix and influence signalling activity. Members of the small leucine-rich proteoglycans (SLRPs), biglycan and decorin, are major constituents in dentine (Waddington *et al.*, 2003a). These SLRPs have proposed roles in modulation of growth factor bioactivity and facilitating their sequestration within biological matrices, thereby protecting them from proteolysis, extending their extracellular half-life and regulating release to the cell (Baker *et al.*, 2009). In addition, biglycan has been described to directly stimulate bone formation *via* dual signalling mechanisms of the BMP/TGFβ and the canonical Wnt/β-catenin pathways and hence has been proposed as a promising therapeutic in bone related diseases involving defects in these pathways (Nikitovic *et al.*, 2012; Nastase *et al.*, 2012).

Both *in vitro* and *in vivo* studies have demonstrated an ability for dentine matrix components to drive mineralised tissue repair, and thus DDM may be regarded as a physiologically optimised bioactive matrix that is able to drive wound repair responses, and thus may have a therapeutic application for stimulating bone repair and regeneration. However, whilst the presence of prime growth factors with proposed roles in bone repair can presumed to be present, a fuller characterisation of the growth factor profile DDM contains still remains unknown. Further, the importance of other extracellular matrix components, such as the SLRPs, for synergistically enhancing growth factor activity has not previously been established as a key contributor to DDM's bioactivity. Using human derived tissue and cells, this study quantified the physiological growth factor levels in DDM able to evoke an osteogenic response, in addition to confirming the presence of biglycan and decorin. Bioactivity of DDM, including determining dose effects, was examined, in order to dissect its regulatory activity on key biological events that are prerequisite for successful bone repair; namely, MSC migration, promoting cell survival and regulating proliferation, osteoblast differentiation and deposition of a mineralised matrix. Through fractionation of growth factors and immuno-depletion of decorin and biglycan matrix components, the importance of these components for driving osteogenesis of human BMMSCs was evaluated.

Materials and Methods

Preparation of demineralised dentine matrix (DDM)
Human teeth (representing healthy tissue from 10 molars, pre-molars, canines and incisors) were obtained from Cardiff University School of Dentistry "Toothbank," with patient informed consent in accordance with ethical approval granted by South East Wales Research Ethics Committee, (12/WA/0289). Enamel, cementum and soft tissues of the pulp and attached gingiva were dissected away and the remaining dentine (7.5 g) was powdered using a percussion mill cooled in liquid nitrogen. DDM was extracted from dentine powder using 7.5 % EDTA diNa$^+$, as previously described by Lee *et al.* (2015). Soluble extracts were recovered by centrifugation at 1,100×*g* for 10 min, dialysed against double distilled water (ddwater) at 4 °C, lyophilised and stored at −20 °C (total of 0.5 g DDM produced for all subsequent experiments described). DDM aliquots were reconstituted to 1 mg/mL in tris-buffered saline (TBS) and sterile filtered through a 0.22 μM filter. Protein concentration was determined using a Pierce® BCA Assay Kit (Thermo Fisher Scientific, Newport, UK).

Characterisation of DDM

SDS-PAGE and Western blot analysis
DDM samples were separated by electrophoresis using 4-15 % Mini-PROTEAN® TGX pre-cast gels (Bio-Rad, Hemel Hempstead, Herts, UK) with running buffer 25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3, at 200 V. A Silver Stain Plus™ kit (Bio-Rad, Hemel Hempstead, Herts, UK) was used to visualise protein. Western blot analysis of separated protein components were conducted as described by Sadaghiani *et al.* (2016). Details of antibodies

Table 1. Details of primary antibodies, corresponding secondary antibodies and respective blocking peptides (used to confirm specificity of antibody binding) for immuno detection in Western blot analysis and immuno-precipitation protocols. (All antibodies and blocking peptides from Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA; N/A not available).

Primary antibody (Ab)	Primary Ab Dilution	Secondary Ab	Secondary Ab dilution	Blocking peptide
TGF-β1 (V)	1:200	Anti-rabbit HRP (sc-2030) –	1:25,000	TGF-β1 (V)
BMP-2 (N-14)	1:500	Anti-goat HRP (sc-2020)	1:50,000	BMP-2 (N-14)
VEFG (A-20)	1:200	Anti-rabbit HRP (sc-2030)	1:25,000	VEFG (A-20)
Biglycan (L-15)	1:500	Anti-goat HRP (sc-2020)	1:50,000	N/A
Decorin (N-15)	1:300	Anti-goat HRP (sc-2020)	1:50,000	N/A

and blocking peptides are described in Table 1. Negative controls were performed by incubating primary antibodies with 10× excess of respective blocking peptide for 1 h prior to incubation with the membrane.

ELISA

DDM was reconstituted in α-MEM (containing ribonucleosides and deoxyribinucleosides) (Thermo Fisher Scientific, Newport, UK) and growth factors quantified using the following commercially available kits; Human TGF-β1 Platinum ELISA kit (eBioscience, Altrincham, Ches., UK); Human IGF-1 Quantikine® ELISA kit (R&D Systems, Abingdon, Oxon, UK); Human BMP-4 and Human BMP-7 ELISA kits respectively (Sigma-Aldrich, Poole, UK); BMP-2, VEGF, PDGF-BB and FGF-2 using respective Human Mini ELISA kits (PeproTech, London, UK). Assays were performed in triplicate on three separate occasions (two separate occasions for BMP-4 and BMP-7 where lack of material prevented analysis for detection of low growth factor levels).

Fractionation of DDM

Heparin affinity chromatography

DDM was reconstituted in binding buffer (0.01 M Tris-HCl / 0.1 M NaCl, pH 7, in 18.2 MΩ water) and passed through a sterile 0.22 μM syringe filter. Protein concentration was determined. 1.8 mg was applied to a Heparin HiTrap column, incorporated into an AKTA Purifier FPLC system (both GE Healthcare Life Sciences, Amersham, Bucks, UK) over 15 mL at 1 mL/min. Flow through eluent (FT) was collected as one fraction. Bound DDM components were eluted by application of a linear 0.1-3 M NaCl gradient, over 12 column volumes at 2 mL/min. 2.5 mL fractions were collected and subsequently pooled into EL1 and EL2, based on the 280 nm absorbance profile. Pooled fractions were dialysed against ddwater, lyophilised and re-suspended in TBS. Protein concentration in each fraction was determined and expressed as a percentage of original protein added to the column. TGFβ-1 and BMP-2 concentrations in each fraction were determined by ELISA and the presence of biglycan and decorin by Western blot analysis.

Immuno-precipitation of decorin and biglycan from DDM

Immunoprecipitation was achieved using a Pierce® Crosslink IP Kit (Thermo Fisher Scientific, Newport, UK), according to manufacturer's protocol. Columns were cross-linked with either 10 μg biglycan (sc-27936) or decorin (sc-22613) antibody (both from Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). 700 μg DDM was applied to respective antibody-loaded columns and incubated overnight. Biglycan / decorin depleted DDM, followed by bound material, was eluted from the column following manufacturer's protocol. Eluents were dialysed against ddwater, lyophilised and re-suspended in TBS. Protein concentration was determined and expressed as a percentage of DDM applied to the column.

Cell source, preparation and culture

BMMSCs were obtained commercially (Lonza, Slough, UK; from a 30-year-old male donor). Cells were seeded at 5,000 cells/cm^2 and cultured in basal medium (α-MEM containing ribonucleosides and deoxyribinucleosides (Thermo Fisher Scientific, Newport, UK), 10 % foetal calf serum (Invitrogen, Thermo Fisher Scientific, Newport, UK), 100 μM L-ascorbic-2-phosphate (Sigma-Aldrich, Poole, UK), 1× Antibiotic/Antimycotic (Sigma-Aldrich, Poole, UK)), 37 °C, 5 % CO_2. At 70-80 % confluence, a single cell suspension was produced using Accutase (PAA Laboratories GmbH, Pasching, Austria) and immature cells were selected by preferential adherence to fibronectin (human plasma; Sigma-Aldrich, Poole, UK) over 20 min, as described previously by Lee *et al.* (2015). Preferential selection of fibronectin-adherent BMMSCs was performed to increase the homogeneity of the cell population and reduce the effects of donor variability when replicating these experiments. Fibronectin adherent cells (FN-BMMSCs) and BMMSCs were culture expanded in the above media.

MSC characterisation

Population doublings (PDs)

At each passage, cell counts were determined and PDs calculated, proportional to cell numbers initially seeded:

$$PD = \frac{log_{10}total\ cell\ count\ obtained - log_{10}total\ cell\ count\ reseeded}{log_{10}2}$$

Cells were reseeded at 4,000 cells/cm^2.

Reverse Transcription PCR

BMMSCs and FN-BMMSCs were cultured until 90-100 % confluent. RNA was extracted using an

RNeasy kit (Qiagen, Hilden, Germany). cDNA was synthesised from 1 µg of total RNA using the M-MLV reverse transcriptase system (Promega, Southampton, UK,), amplified within a Thermal Cycler at 70 °C for 5 min. Total human RNA (Clontech, Saint-Germain-en-Laye, France) or nuclease free water to replace sample RNA served as respective positive or negative controls. Expression of purported mesenchymal, embryonic and haematopoietic stem cell markers was examined using a standard RT-PCR protocol with forward and reverse primers, detailed in Table 2, and the following cycling conditions: 1 initial denaturation at 95 °C, 4 min; 35 cycles of 95 °C, 1 min and 72 °C, 1 min; 1 final extension at 72 °C, 10 min. RT-PCR products were separated on 2 % agarose/ethidium bromide gels and visualised by UV light.

β-galactosidase staining

MSCs, cultured to suspected senescence as indicated by PD < 0.5, and pre-senescent cells were analysed. β-galactosidase staining was performed using a Senescent Cells Histochemical Staining Kit (Sigma-Aldrich, Poole, UK).

Culture of FN-BMMSCs in the presence of DDM and fractions

For fractionated DDM samples, protein concentration added to culture media was adjusted to represent the percentage of total DDM as calculated above (*i.e.* percentage of 10 µg/mL).

MTT Cell expansion assay

FN-BMSCs (10 PDs) were seeded into 96-well plates at 4,000 cells/cm^2 in 5 % foetal bovine serum (FBS) culture medium, supplemented with 0-10 µg/mL DDM or relative proportion of fractionated sample. Cell expansion was determined using a MTT assay (Sigma-Aldrich, Poole, UK) over a 0-96 h culture period.

Caspase apoptosis assay

FN-BMSCs (14.5 PD) were seeded into white-walled, optically clear 96-well plates at 4,000 cells/cm^2 in 10 % FBS culture medium supplemented with 0-10 µg/mL DDM. Apoptotic cells were detected over 48 h using a Caspase-Glo® 3/7 Assay (Promega, Madison, WI, USA).

Cellular migration assay

Cell migration through collagen was measured using Boyden chamber assay system. 52.54 µL type 1 collagen (supplied 3.7 mg/mL in 0.02 N acetic acid; Sigma-Aldrich, Poole, UK) was added to 1 mL α-MEM and neutralised with 10.58 µL 0.1 N NaOH. The uppermost surface of 24-well tissue culture inserts (8 µM pores; Greiner Bio-One, Stonehouse,

Table 2. Details of forward and reverse primer sequences and cycling conditions used for RT-PCR and qPCR. GAPDH was used as reference gene.

Gene product	Primer sequence: 5′-3′	Annealing temperature (°C)	Product length (bp)	Application
GAPDH	**F**: TTCTTTTGCGTCGCCAGCCGA **R**: GTGACCAGGCGCCCAATACGA	55	96	RT PCR / qPCR Housekeeping gene
CD105	**F**: GAAACAGTCCATTGTGACCTTCAG **R**: GATGGCAGCTCTGTGGTGTTGACC	55	344	RT PCR MSC marker
CD90	**F**: ATGAACCTGGCCATCAGCATCG **R**: CACGAGGTGTTCTGAGCCAGCA	55	425	RT PCR MSC marker
CD73	**F**: GTCGCGAACTTGCGCCTGGCCGCCAAG **R**: TGCAGCGGCTGGCGTTGACGCACTTGC	55	352	RT PCR MSC marker
CD146	**F**: TGTGTAGGGAGGAACGGGTA **R**: TGGGACGACTGAATGTGGAC	55	101	RT PCR MSC marker
CD34	**F**: GCTTTGCTTGCTGAGTTTGC **R**: CCATGTTGAGACACAGGGTGC	55	180	RT PCR Haemopoietic stem cell marker
CD45	**F**: CCCCACTGATGCCTACCTTA **R**: ATGCACCTCATTGTTTGTGC	55	238	RT PCR Haemopoietic stem cell marker
Oct4	**F**: GGTGGAGAGCAACTCCGAT **R**: ATGGTCGTTTGGCTGAATACC	55	246	RT PCR Embryonic MSC marker
Nanog	**F**: AGGTCCCGGTCAAGAAACAG **R**: CCCTGCGTCACACCATTG	55	235	RT PCR Embryonic MSC marker
RunX2	**F**: GGTTAATCTCCGCAGGTCACT **R**: CCCTCTGTTGTAAATACTGCTTGC	55	80	qPCR / RT PCR Early osteoblast differentiation marker
CO-L1A1	**F**: TGCTGTTGGTGCTAAGGG **R**: GCAATACCAGGAGCACCATTG	55	177	qPCR Matrix deposition marker
OC	**F**: GGCAGCGAGGTAGTGAAGAG **R**: CTCACACACCTCCCTCCT	55	102	qPCR Late osteoblast differentiation marker

Glos., UK) were coated with 10 µg of type I collagen in solution, left to solidify at 37 °C overnight and then air dried for 1 h at room temperature. FN-BMMSCs (12.6 PDs) were serum starved for 24 h and re-seeded at 10,000 cells/cm^2 in 200 µL/well in serum-free culture medium onto the collagen-coated inserts. Inserts were placed into the lower chamber containing 600 µL/well serum-free medium supplemented with 0-10 µg/mL DDM and incubated at 37 °C, 5 % CO_2. After 20 h medium in the lower compartment was replaced with 450 µL of serum-free culture medium containing 8 µM Calcein-AM (Sigma-Aldrich, Poole, UK) and incubated at 37 °C for 45 min. This culture medium was then replaced with 500 µL of 0.05 % Trypsin-EDTA (Sigma-Aldrich, Poole, UK) to detach cells over 10 min at 37 °C. 200 µL of Trypsin solution was removed from each well and fluorescence read using 485 nm excitation/520 nm emission wavelengths.

Osteogenic differentiation

FN- BMMSCs (15-17 PD) were seeded at 10,000 cells/cm^2 and cultured in α-MEM basal medium supplemented with 0-10 µg/mL DDM or equivalent fractionated sample at 37 °C, 5 % CO_2. Basal medium supplemented with 10 mM β-glycerophosphate and 10 nM dexamethasone (both Sigma-Aldrich, Poole, UK) provided a positive osteogenic control.

Alizarin Red Staining

After 28 d, cultured cells were washed twice with PBS, fixed in 10 % formaldehyde (Sigma-Aldrich, Poole, UK), 30 min, washed twice in PBS and stained with 2 % (w/v) Alizarin Red S (pH 4.1-4.3) (Sigma-Aldrich, Poole, UK) for 20 min. Cells were washed with ddwater and digital images captured with a light microscope.

Quantitative real-time PCR

Gene expression for osteogenic related markers RunX2, osteocalcin (OC) and Col1A1 was assessed. RNA was extracted from cultured cells and converted to cDNA by reverse transcription as described above. For qPCR analysis, cDNA was added to primer sets (Table 2) and PrecisionFAST Master Mix pre-mixed

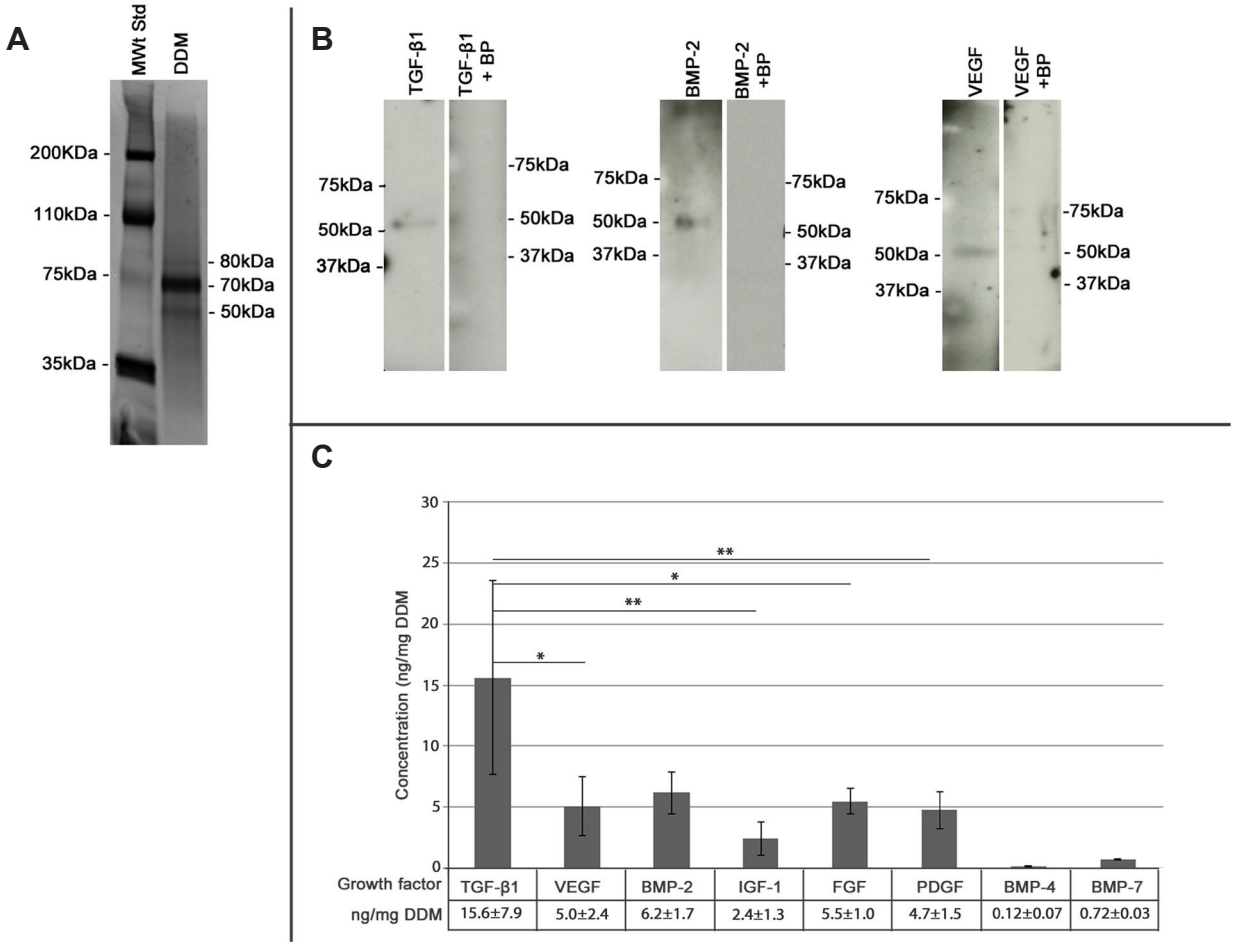


Fig. 1. Assessment of growth factor composition present in DDM. (**A**) Separation of DDM by SDS PAGE with strong silver staining protein bands evident at 80, 70 and 50 kDa. (**B**) Immuno-detection of TGF-β1, BMP-2 and VEGF within DDM, with all detected within the 50 kDa protein band. (**C**) Quantification of growth factors present in DDM using respective ELISA assays. From standard curves, growth factors were calculated to be present within the DDM in low ng to pg/mL concentration range, with TGF-β1 the most abundant growth factor. ELISAs were performed in triplicate on three separate occasions *= $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

with SYBR green (Primer Design Ltd, Eastleigh, Hamps, UK). qPCR was performed using QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Newport, UK) with the following cycling reaction conditions: 1 initial denaturation at 95 °C for 20 s; 40 cycles of denaturation for 1 s, annealing for 20 s. GAPDH was the internal reference gene.

Statistical analysis

For all assays performed above, triplicate samples were analysed for each sample group examined, and assays were repeated on three separate occasions. Statistical analyses to determine analysis of variance between sample groups were performed by One-Way ANOVA with a *post-hoc* Tukey test using Instat GraphPad software (GraphPad, La Jolla, CA, USA).

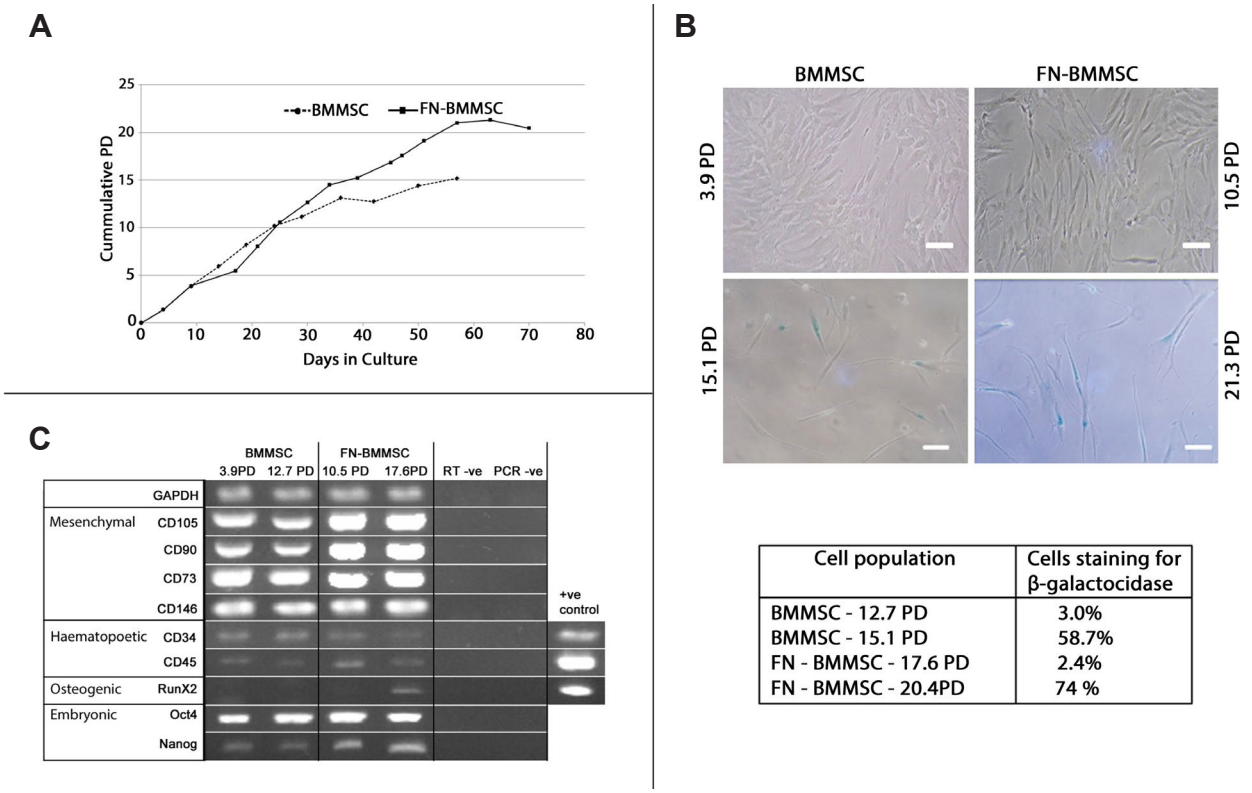
Results

Quantification of growths factors in DDM

Separation of DDM by SDS PAGE indicated the presence of protein material from 250 kDa to 6 kDa, with strong bands at approximately 80 kDa, 70 kDa and 50 kDa (Fig. 1**A**). Subsequent Western blot analysis of separated gels, indicated immuno-positivity for TFG-β1, BMP-2 and VEGF at 50 kDa. Pre-incubation of the primary antibody with their respective blocking peptide confirmed specificity for the antibody used (Fig. 1**B**). ELISA quantification of growth factors revealed TGF-β1 as the most abundant growth factor (15.6 ± 7.9 ng/mg DDM; n = 3). FGF-2, BMP-2, PDGF, VEGF and IGF-1 demonstrated intermediate abundance (6.2-2.4 ng/mg; n = 3). BMP-4 and BMP-7 represented the lowest abundant factors (approximately 120-72 pg/mg DDM; n = 2 as insufficient material available to accurately detect growth factors present at pg levels).

Isolation of immature FN-BMMSC cells from BMMSCs

Cells used in this study were selected for adherence to fibronectin to isolate the more immature MSC population (FN-BMMSCs; Lee *et al.*, 2015; Jones and Watt, 1993), to reduce heterogeneity of the cell population and increase potential for reproducibility on these commercially derived cells, which would likely be obtained from different donors. Both BMMSCs and selected FN-BMMSCs expressed purported MSC markers (Reviewed by Mafi *et al.*,



Cell population	Cells staining for β-galactocidase
BMMSC - 12.7 PD	3.0%
BMMSC - 15.1 PD	58.7%
FN - BMMSC - 17.6 PD	2.4%
FN - BMMSC - 20.4PD	74 %

Fig. 2. Comparison of MSC characteristics of commercial BMMSCs and the same cells selected by preferential fibronectin adherence to select a more immature cell population, FN-BMMSC. (**A**) Following culture expansion FN-BMMSCs demonstrated an ability to proliferate to approximately 20 PD whilst proliferation of parent BMMSCs started to slow at approximately 13 PDs. (**B**) RT-PCR demonstrated the expression of all classical MSC markers by both populations; an increase in the embryonic marker Nanog was observed for FN-BMMSC. (**C**) Culture expansion induced senescence within both cell populations as determined by blue cells staining for β-galactosidase; senescence developed at a later PD for FN-BMMSC cells. White scale bars represent 100 μM.

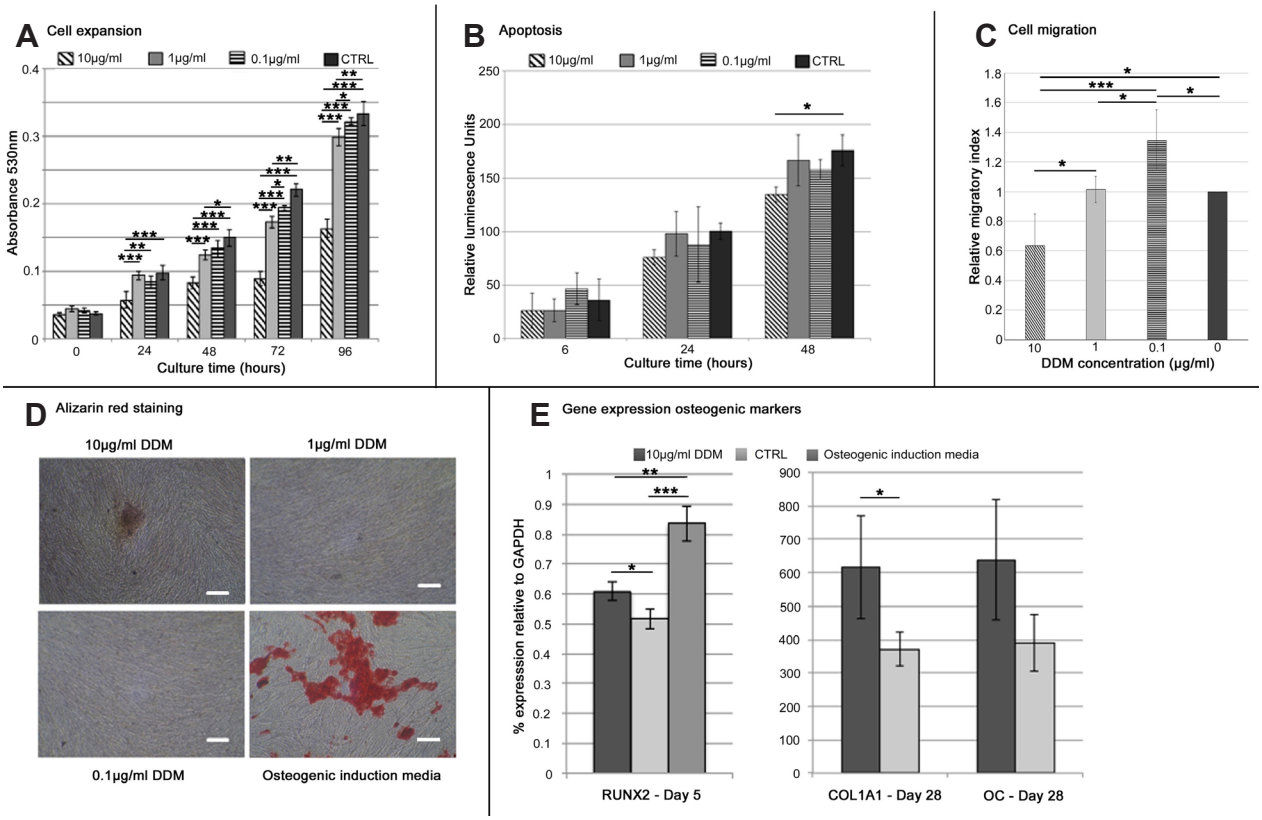


Fig. 3. (**A**) DDM reduced the expansion of FM-BBMSCs, which appeared to be dose dependent, with significant reduction for cells supplemented with 10 µg/mL compared to non-supplemented cells observed at all time points and significance obtained for cells with 1 µg/mL DDM after 48 h. (**B**) 10 µg/mL DDM was the only concentration observed to significantly inhibited cell apoptosis as demonstrated by a reduction in the fluorescence read-out. (**C**) 10 µg/mL DDM also inhibited the migration of cells through a collagen matrix, whilst 0.1 µg/mL DDM promoted cell migration, compared to the non-supplemented control. (**D**) 10 µg/mL DDM supplementation was sufficient to induce deposition of mineralised nodules by the FM-BMMSCs as visualised by Alizarin Red staining and (**E**) the significant increase in gene expression for RunX2, Col1A1 and OC as determined by qPCR. Osteogenic induction media represented a positive control. n = 3 for all assays identifying cellular behaviour. *= $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. White scale bars represent 100 µm.

2011) CD105, CD90, CD73 and CD146, embryonic marker Oct4 and were negligible for haemopoietic markers CD34, CD45 (Fig 1**B**). The selection of more immature cells was indicated by an increased proliferative lifespan for FN-BMMSCs, which achieved >17 PDs in long-term culture (Fig. 2**A**), with 75 % of cells observed as senescent at 20.4 PDs (Fig. 2**C**); by comparison proliferation of BMMSCs slowed to <0.5 PD at 12 PD and demonstrated 58.7 % senescent cells at 15.1 PD. In addition, FN-BMMSCs expressed the embryonic stem cell marker Nanog (Mafi *et al.*, 2011), which was perceived as less pronounced in BMMSCs (Fig. 2**B**), indicating that cells expressing an immature MSC phenotype remained following fibronectin selection.

Influence of DDM on FN-BMMSC cell behaviour

FN-BMMSCs supplemented with 10 µg/mL DDM significantly reduced cell expansion, apoptosis, cell migration (Fig. 3**A-C**) and was the minimal concentration able to induce formation of mineralising nodules (Fig. 3**D**), confirmed by increased gene expression of osteogenic markers RunX2, Col 1A1 and OC (Fig. 3**E**). Lower concentrations of 0.1 and 1 µg/mL DDM failed to produce an osteogenic response, reduced cell expansion to a lesser extent, albeit statistically significant at later time points, and had no effect on apoptosis. When the influence of DDM was assessed on cell migration, an inverse dose effect was observed. 0.1 µg/mL DDM stimulated migration of cells through the collagen matrix, whilst no influence in cell migration was observed in the presence 10 µg/mL DDM.

Influence of fractionating growth factors from DDM using heparin affinity chromatography

Growth factors were fractionated from DDM by virtue of their affinity to heparin (Fig. 4). 280 nm absorbance elution profiles indicated that the majority of the DDM constituents failed to bind to the column, passing into the flow through (FT) eluent. Consistent with the low concentration of growth

factors quantified in DDM, a small eluting peak was observed in fraction EL1 and minimal protein was detected in EL2 (Fig. 4**B**). ELISAs were performed, as a within assay triplicate, on one occasion (due to limitation of material) but indicated that over half of the BMP-2 and approximately 21.6 % of TGF-β1 was present in the FT fraction (Fig. 3**C**). Western blot analysis of the FT demonstrated both biglycan and decorin as present (Fig. 4**D**). Insufficient material prevented similar analysis of biglycan and decorin in EL1 and EL2. Approximately 36 % of total TGF-β1 present in DDM bound heparin, eluting in EL2, whilst approximately 7 % of BMP-2 was present in EL1 and EL2 combined (Fig. 4**C**).

Whilst total fractionation of growth factors was not achieved, fractionation influenced activity on FM-BMMSCs (Fig. 5; n = 3 for all analysis on cell behaviour). Compared to supplementation with whole DDM, constituents in eluted fractions appeared to increase cell expansion to levels approaching rates observed for non-supplemented cells, although a statistically significant difference was achieved when comparing whole DDM with EL1 (Fig. 5**A**). EL1 and EL2 demonstrated similar expansion rates to those observed for non-supplemented cells (Fig. 5**A**). Compared with whole DDM, fractionated samples also had a reduced osteogenic potential as demonstrated by a lack of stimulation of RunX2 gene

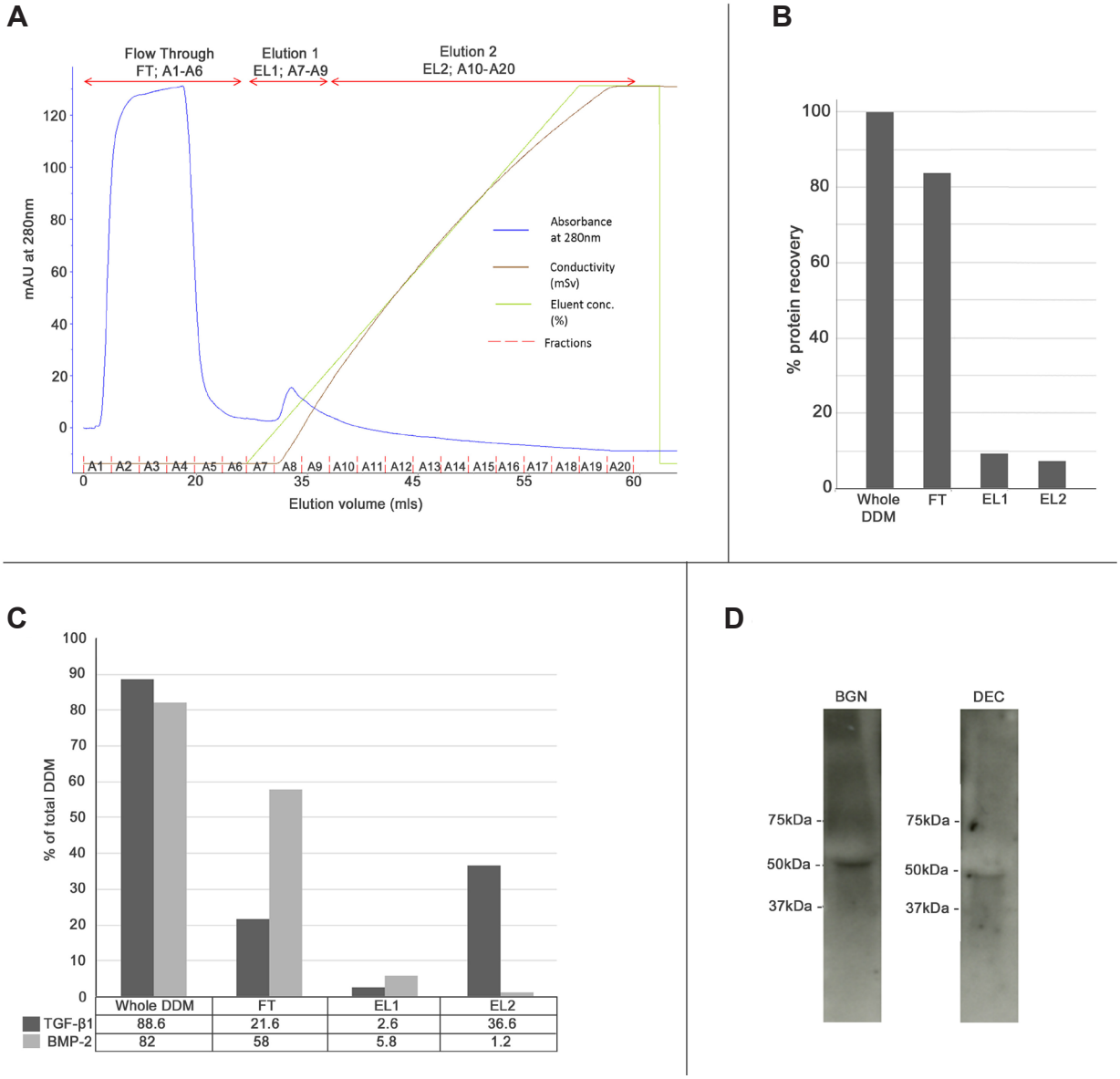


Fig. 4. Fractionation of growth factors using heparin affinity chromatography. (**A**) DDM was applied to heparin column and based on protein elution profiles, measuring 280 nm absorbance, eluting fractions were pooled to produced non-binding flow through (FT) fraction and fractions with increasing binding potential of EL1 and EL2. (**B**) Protein concentration was measured for each fraction and expressed as a percentage of the whole DDM, indicating low level binding of growth factors to the column, consistent with the low concentration levels of growth factors present in DDM. (**C**) Growth factors in each fraction were estimated using ELISA (performed in triplicate on two separate occasions; n = 2 due to insufficient material) and (**D**) the presence of biglycan and decorin confirmed by Western blot analysis using the appropriate primary antibodies.

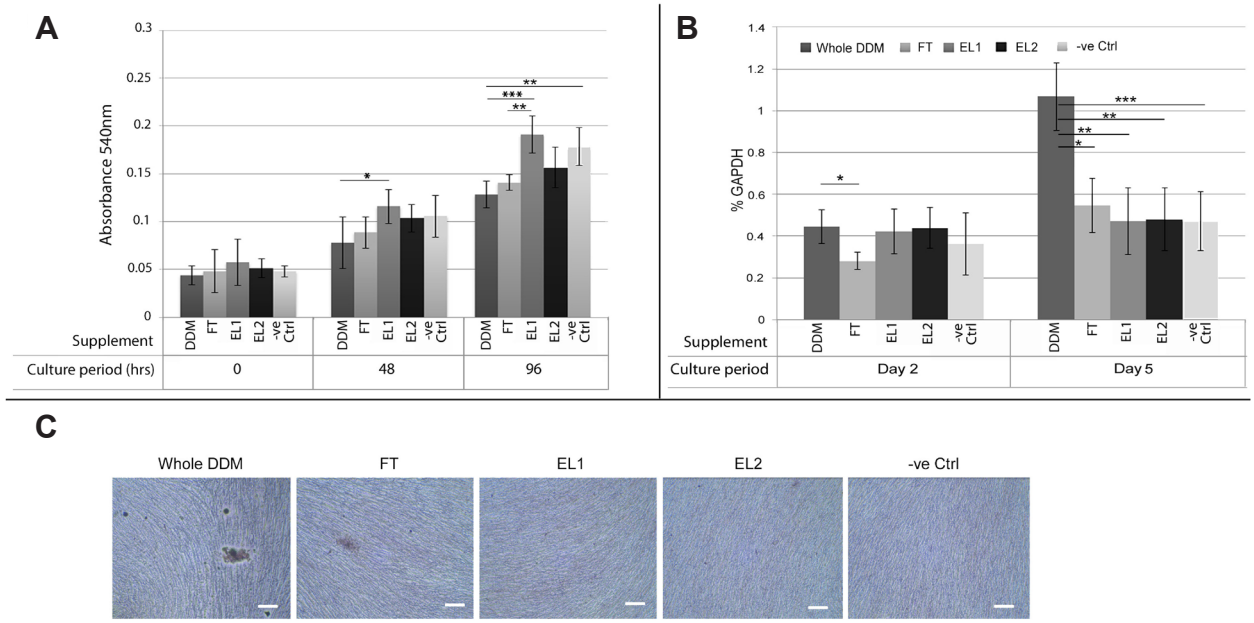


Fig. 5. The influence of fractions following heparin affinity chromatography on FM-BMMSCs. (**A**) All fractions appeared abolish the observed influence of whole DDM in decreasing cell expansion when compared to non-supplemented control cells, with statistical significance achieved of a higher cell expansion observed for cells supplement with EL1 compared with whole DDM. (**B**) Whole DDM increased the gene expression of RunX2 in FM-BMMSCs. For all fractions no increase in RunX2 was observed. (**C**) Following continued culture in the presence of the fractionated samples to 28 d, small nodules with faint staining for Alizarin Red were observed for FT fraction, whilst no mineralising nodule formation was observed for EL1 and EL2. Supplementation with whole DDM demonstrated reproducible results compared to Fig. 2. All cell based assays were performed in triplicate on three separate occasions. *= $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. White scale bars represent 100 μm.

expression (Fig. 5**B**) and no or weak mineralised nodule formation, visualised by Alizarin red (Fig. 5**C**).

Immuno-precipitation of biglycan and decorin from DDM

Western blot analysis confirmed the single depletion of biglycan or decorin from the DDM (−BGN and −DCN respectively). Dual depletion of both decorin and biglycan from DDM was also achieved (−BGN/DCN). Analysis of the immuno-precipitated products (+BGN, +DCN and +BGN/DCN) confirmed the co-precipitation of TGF-β1, which was not detected in the immuno-depleted DDM counterparts (Fig. 6**A**). Immuno-depleted −BGN or −DCN abolished the inhibitory effect whole DDM produced on cell expansion (observed when DDM is compared with non-supplemented control (Fig. 6**B**)). Immuno-precipitated +BGN and +DCN increased cell expansion compared to the non-supplemented control. Immuno-depletion for −BGN/DCN produced results similar to DDM, whilst the immuno-precipitated product +BGN/DCN produced cell expansion rates similar to the non-supplemented control. This study also considered the effect of immuno-precipitation on osteogenic potential as judged by gene expression for RunX2 (Fig. 6**C**) and deposition of mineralising nodules staining with Alizarin Red (Fig. 6**D**). Immuno-depleted −BGN and dual depletion for −BGN/DCN abolish osteogenic potential; immuno-precipitated +BGN or +BGN/DCN produced an osteogenic effect, albeit less than that observed for whole DDM; immuno-depleted −DCN slightly reduced osteogenic potential, and +DCN failed to induce either RunX2 expression or deposition of mineralising nodules.

Discussion

In vivo, dentine matrix components have been observed to promote mineralised dentine tissue repair within the tooth (Reis-Filho *et al*., 2012; Chun *et al*., 2011) and in promoting bone repair (de Oliveira *et al*., 2013; Gomes *et al*., 2008). DDM can thus be described to represent a physiologically optimised model matrix for promoting osteogenic signalling. A major aim of this study was to quantify in DDM the physiological concentrations of potential candidate growth factors recognised to act synergistically to elicit osteogenic potency (Kanczler *et al*., 2010; Tachi *et al*., 2011; Simmons *et al*., 2004). Notably TGF-β1 DDM was found to be the most abundant growth factor (15.6 ng/mg), with levels more than double those identified for BMP-2, FGF, PDGF and VEGF. The molecular weight of TGF-β1 (52 kDa) suggests

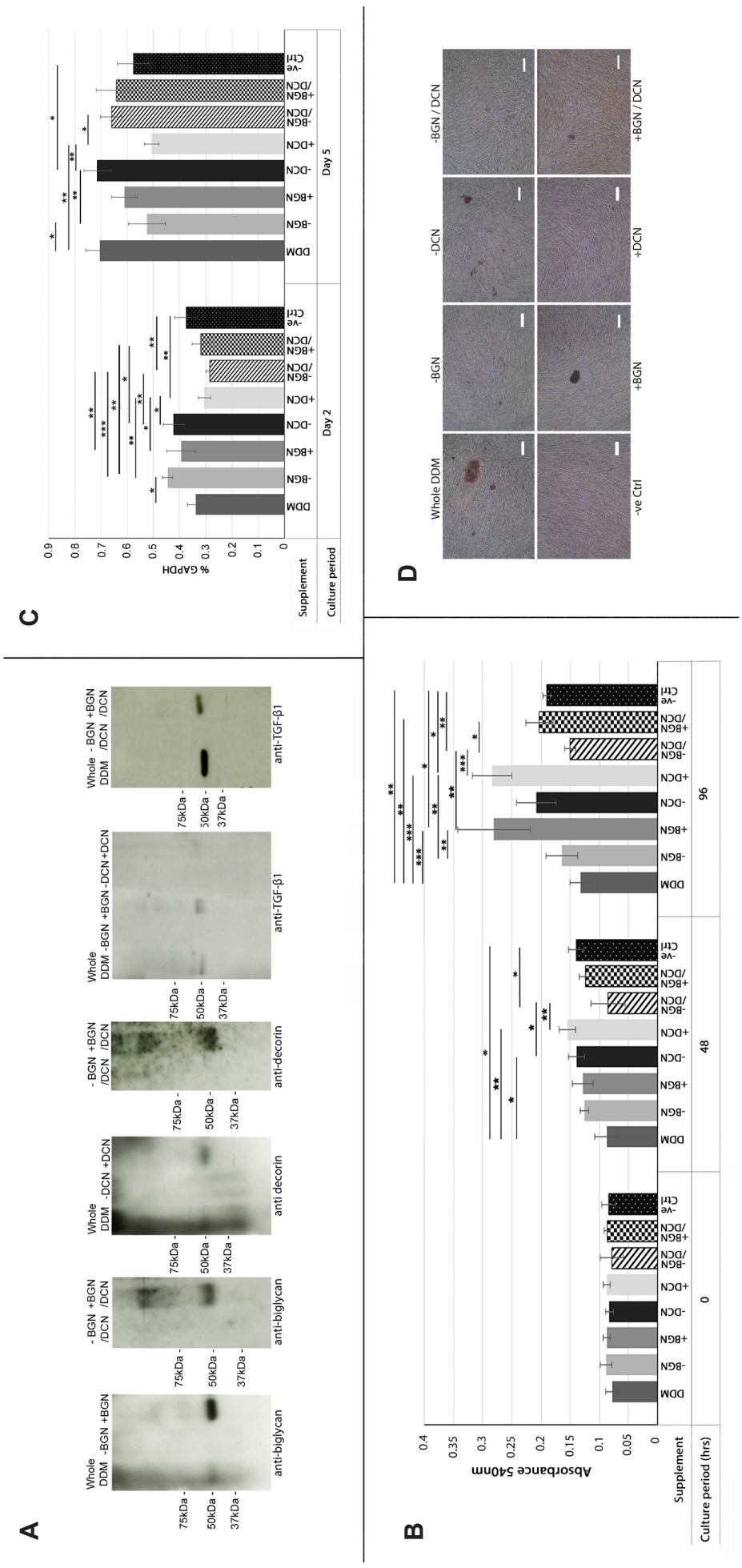


Fig. 6. Influence of immuno-precipitation of biglycan and / or decorin from DDM of cellular behaviour of FMBMMSCs. (**A**) Western blot analysis confirming the complete depletion of biglycan (–BGN) or decorin (–DCN) or dual depletion (–BGN/DCN) from DDM. Analysis indicated that TGF-β1 co-precipitated with the +BGN, +DCN or the dual +BGN/DCN precipitated products. (**B**) The influence of the depleted and precipitated products on cell expansion, measured by MTT assay. DDM reduced cell expansion rates which was not observed with –BGN, –DCN or –BGN/DCN. +BGN and +DCN enhanced cell expansion greater than the non-supplemented negative control. (**C**) Gene expression of RunX2. DDM enhanced RunX2 expression compared to negative control. –BGN, +BGN, +DCN failed to stimulate RunX2 expression. –DCN depletion did not abolish the increase in RunX2 expression also produced by whole DDM. (**D**) Visualisation of mineralising nodule formation stained with Alizarin red. +BGN, –DCN and –BGN/DCN produced mineralising nodules, although smaller compared to whole DDM. –BGN, +DCN and –BGN/DCN failed to stimulate mineral nodule formation. All cell based assays were performed in triplicate on three separate occasions. *= $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

it is present as the unprocessed precursors (Gentry *et al.*, 1988), although this form is predicted to be biologically active (Baillie *et al.*, 1996). TGF-β1 can induce new bone formation when injected into critical size defects (Blom *et al.*, 2001), where it acts co-operatively with BMPs to induce osteogenic differentiation (Lee *et al.*, 2003; Lee *et al.*, 2000). This is consistent with the observation that both growth factors can activate independently RunX2, although only BMP-2 activates the osteogenic pathway by Dlx-5 expression (Lee *et al.*, 2003). TGF-β can also induce BMP synthesis in the osteoprogenitor cells (Bostrom, 1998). TGF-β1 also signals synergistically with growth factors such as FGFs (Mansukhani *et al.*, 2000) and IGFs (Hughes *et al.*, 2006), where collectively they have been attributed prominent roles in the migration of MSC and macrophages to the wound healing site, to induce MSC proliferation and stimulate the production of a collagenous osteoid matrix. Other synergistic signalling networks are also observed in DDM. VEGF is recognised as an important mitogen regulating budding of new endothelial cells for re-vascularisation (Dimitriou *et al.*, 2005). Its action has been shown to be enhanced by PDGF and basic FGF (Al-Aql *et al.*, 2008), which are also identified in DDM in the present study.

Within our study, 10 μg/mL of DDM was the minimal concentration capable of inducing an osteogenic response, which correlated with a decreased cell proliferation. At this concentration, DDM significantly reduced apoptotic cell numbers, indicating a role for promoting cell survival. However, at low concentrations of 0.1 μg/mL, DDM presented a chemo-attractant role, inducing migration of cells through a collagen matrix, whilst 10 μg/mL inhibited cell migration. Similar actions for DDM preparations have been noted for dental pulp stem cells (Lee *et al.*, 2015; Gonçalves *et al.*, 2016; Liu *et al.*, 2016) although it should be noted that for these studies the demineralised dentine extract was prepared using different protocols that varied around the theme of demineralising the mineralised tissue and thus would lead to DDM of different composition to that identified in the present study. As a direct comparison, for DDM prepared following the same protocol (Lee *et al.*, 2015), the up-regulation of bone forming markers in dental pulp stem cells has been shown to be induced with DDM at 1 μg/mL, with 10 μg/mL DDM significantly increasing cell proliferation, thus highlighting differential effects of DDM on cells from different tissue sources. Within the culture media in our study, 10 μg/mL equates to approximately 110 pg/mL of TGF-β1 and 50 pg/mL of BMP-2, FGF and PDGF. One hundred-fold lower concentrations induce MSC migration. These levels are multiple times lower than the plethora of *in vitro* studies investigating one or two growth factors. The results emphasise a strong therapeutic potential for these physiological levels of growth factors when considering placement of DDM in the following bone healing site scenario *in vivo*. DDM first promotes the migration of BMMSCs along a chemo-attractant gradient of increasing concentration of stimulatory factors. Migratory potential is subsequently negated when the cell reaches the wound healing site where the higher concentration of DDM enhances BMMSC survival, expansion and subsequent osteogenic differentiation. The present study also indicated how the ratio of growth factors, operating *via* various additive or opposing regulatory signalling pathways, are important in producing an overall osteogenic effect. When DDM was fractionated, although heparin binding fractions contained TGF-β1 and BMP-2 (EL1 and EL2), supplementation of FN-BMMSCs with these fractions failed to induce an osteogenic effect. Unbound DDM (FT), also contained reduced levels of TGF-β1 and BMP-2 (21.6 % and 58 % of total present in whole DDM), and also produce less intensely stained mineralising nodules.

This study additionally investigated the bioactive contribution of decorin and biglycan to the DDM, as matrix components able to bind TGF-β1 and BMP-2 (Baker *et al.*, 2009). Indeed, immuno-precipitation of either biglycan or decorin from the DDM was accompanied by the co-precipitation of TGF-β1. Our results are notable for indicating a strong osteogenic inductive role for biglycan, but not for decorin. DDM depletion for biglycan abolished osteogenic differentiation, but precipitated biglycan supported an osteogenic response by the FN-BMMSC cells. Decorin-depleted DDM produced an osteogenic response, but precipitated decorin did not. These observations mirror knock-out studies assessing roles for biglycan and decorin. *In vivo*, biglycan$^{-/-}$ mice experience reduced bone growth and skeletal mass compared to wild-type animals (Xu *et al.*, 1998), in addition to reduced callus size and less woven bone at the fracture site (Berendsen *et al.*, 2014). Decorin-depletion *in vivo* did not affect bone quality, although double depletion of both decorin and biglycan produced mice with severe bone defects (Young *et al.*, 2006). *In vitro*, transfection of MC3T3-E1 cells with biglycan enhances matrix mineralisation, whilst mineralisation is depressed in biglycan-KO cells (Parisuthiman *et al.*, 2005). These studies have led to the proposal that biglycan possesses modulatory effects on growth factor activities by sequestering TGF-β1 and preventing over-activation of signalling (Bi *et al.*, 2005), and enhancing BMP-4 (Chen *et al.*, 2004) and BMP-2 signalling pathways (Berendsen *et al.*, 2011). Furthermore, biglycan has been reported to increase RunX2 transcription as a pericellular ligand binding participant in Wnt signalling (Nikitovic *et al.*, 2012; Nastase *et al.*, 2012).

Within this study, precipitated biglycan or decorin also enhanced cell expansion greater than the non-supplemented control, which may also be attributable to co-precipitating TGF-β1. This result further supports proposed roles for biglycan synthesis in directing proliferation and initiation of osteoblast differentiation in bone derived MSCs/precursor cells (Waddington *et al.*, 2003b; Roberts *et al.*, 2008).

However, the cellular influence of biglycan is likely to be dependent on growth factor concentration and cross-talk from other signalling pathways, and this is demonstrated in the present study. Cell expansion in the presence of both precipitated biglycan / decorin combined, where TGF-β1 concentration would be higher, was similar to non-supplemented control levels. Supplementation with whole DDM, (higher levels of TGF-β1, again), reduces cell expansion compared with the control. Collectively, these results highlight the complex signalling milieu of bone healing. While indicating osteogenic roles for growth factors, which necessitates TGF-β1 co-operative action with BMP-2 and other growth factors, there is also a necessary requirement for other matrix proteins such as biglycan to enhance bioactivity.

Conclusion

This study has identified bioactive utility for DDM in facilitating cell migration, promoting cell survival, regulating proliferation and promoting osteoblast differentiation for enhanced bone repair. Analyses identified optimal growth factor combinations, with prominent roles for TGB-β1, along with biglycan in enhancing an osteogenic response. DDM presents an attractive base for development of a graft material for augmenting bone repair, but significantly one where growth factors are present at pg levels, negating the toxicity issues and potential for ectopic mineralisation encountered in the clinical use of single recombinant forms of growth factors, such as BMP-2. Furthermore, DDM has the capacity to restore compromised signalling environments where *in vivo* studies have already demonstrated the potential of DDM to ameliorate delayed diabetic bone healing (Gomes *et al*., 2008). Levels and ratios of growth factor components are critical to its bioactivity, since fractionation or depletion of components in DDM abrogated osteogenic potential, emphasising that it is the whole DDM matrix that is prerequisite to present an optimised signalling environment to the wound healing site. Of note, DDM contains matrix proteins such as dentine sialoprotein, dentine phosphoprotein, bone sialoprotein, osteopontin, dentine matrix protein-1 and matrix extracellular phosphoglycoprotein (MEPE), with associated proposed signalling roles in the early reparative events (Smith *et al*., 2012); although their roles for a more comprehensive evaluation of DDM's bioactivity have yet to be verified.

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References

Al-Aql ZS, Alagl AS, Graves DT, Gerstenfeld LC, Einhorn TA (2008) Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. J Dent Res **87**: 107-118.

Arana-Chavez VE, Massa LF (2004) Odontoblasts: the cells forming and maintaining dentine. Int J Biochem Cell Biol **36**: 1367-1373.

Axelrad TW, Einhorn TA (2009) Bone morphogenetic proteins in orthopaedic surgery. Cytokine Growth Factor Rev **20**: 481-488.

Baillie R, Coombes RC, Smith J (1996) Multiple forms of TGFβ1 in breast tissues: a biologically active form of the small latent complex of TGFβ1. Eur J Cancer **32A**: 1566-1573.

Baker SM, Sugars RV, Wendel M, Smith AJ, Waddington RJ, Cooper PR, Sloan AJ (2009) TGFbeta/extracellular matrix interactions in dentin matrix: a role in regulating sequestration and protection of bioactivity. Calcif Tissue Int **85**: 66-74.

Berendsen AD, Fisher LW, Kilts TM, Owens RT, Robey PG, Gutkind JS, Young MF (2011) Modulation of canonical Wnt signaling by the extracellular matrix component biglycan. Proc Natl Acad Sci USA **108**: 17022-17027.

Berendsen AD, Pinnow EL, Maeda A, Brown AC, McCartney-Francis N, Kram V, Owens RT, Robey PG, Holmbeck K, de Castro LF, Kilts TM, Young MF (2014) Biglycan modulates angiogenesis and bone formation during fracture healing. Matrix Biol **35**: 223-231.

Bi Y, Stuelten CH, Kilts T, Wadhwa S, Iozzo RV, Robey PG, Chen XD, Young MF (2005) Extracellular matrix proteoglycans control the fate of bone marrow stromal cells. J Biol Chem **280**: 30481-30489.

Blom EJ, Klein-Nulend J, Yin L, van Waas MA, Burger EH (2001) Transforming growth factor-beta1 incorporated in calcium phosphate cement stimulates osteotransductivity in rat calvarial bone defects. Clin Oral Implants Res **12**: 609-616.

Bostrom MP (1998) Expression of bone morphogenetic proteins in fracture healing. Clin Orthop Relat Res **355**: S116-123.

Casagrande L, Demarco FF, Zhang Z, Araujo FB, Shi S, Nör JE (2010) Dentin-derived BMP-2 and odontoblast differentiation. J Dent Res **89**: 603-608.

Chen FM, Zhang M, Wu ZF (2010) Toward delivery of multiple growth factors in tissue engineering. Biomaterials **31**: 6279-6308.

Chen XD, Fisher LW, Robey PG, Young MF (2004) The small leucine-rich proteoglycan biglycan modulates BMP-4-induced osteoblast differentiation. FASEB J **18**: 948-958.

Chun SY, Lee HJ, Choi YA, Kim KM, Baek SH, Park HS, Kim JY, Ahn JM, Cho JY, Cho DW, Shin HI, Park EK. (2011) Analysis of the soluble human tooth proteome and its ability to induce dentin/tooth regeneration. Tissue Eng Part A **17**: 181-191.

de Oliveira GS, Miziara MN, Silva ER, Ferreira EL, Biulchi AP, Alves JB (2013) Enhanced bone formation

during healing process of tooth sockets filled with demineralized human dentine matrix. Aust Dent J **58**: 326-332.

Dimitriou R, Tsiridis E, Giannoudis PV (2005) Current concepts of molecular aspects of bone healing. Injury **36**: 1392-13404.

Finkelman RD, Mohan S, Jennings JC, Taylor AK, Jepsen S, Baylink DJ (1990) Quantitation of growth factors IGF-I, SGF/IGF-II, and TGF-beta in human dentin. J Bone Miner Res **5**: 17-23.

Geiger F, Bertram H, Berger I, Lorenz H, Wall O, Eckhardt C, Simank HG, Richter W (2005) Vascular endothelial growth factor gene-activated matrix (VEGF165-GAM) enhances osteogenesis and angiogenesis in large segmental bone defects. J Bone Miner Res **20**: 2028-2035.

Gentry LE, Lioubin MN, Purchio AF, Marquardt H (1988) Molecular events in the processing of recombinant type 1 pre-pro-transforming growth factor beta to the mature polypeptide. Mol Cell Biol **8**: 4162-4168.

Gomes MF, Destro MF, Banzi EC, Vieira EM, Morosolli AR, Goulart Md (2008) Optical density of bone repair after implantation of homogenous demineralized dentin matrix in diabetic rabbits. Braz Oral Res **22**: 275-280.

Gonçalves LF, Fernandes AP, Cosme-Silva L, Colombo FA, Martins NS, Oliveira TM, Araujo TH, Sakai VT (2016) Effect of EDTA on TGF-β1 released from the dentin matrix and its influence on dental pulp stem cell migration. Braz Oral Res **30**: e131. doi: 10.1590/1807-3107BOR-2016.vol30.0131

Hughes FJ, Turner W, Belibasakis G, Martuscelli G (2006) Effects of growth factors and cytokines on osteoblast differentiation. Periodontol 2000 **41**: 48-72.

Jágr M, Eckhardt A, Pataridis S, Mikšík I (2012) Comprehensive proteomic analysis of human dentine. Eur J Oral Sci **120**: 259-268.

Jones PH, Watt FM (1993) Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell **73**: 713-24.

Kanczler JM, Ginty PJ, White L, Clarke NMP, Howdle SM, Shakesheff KM, Oreffo ROC (2010) The effect of the delivery of vascular endothelial growth factor and bone morphogenic protein-2 to osteoprogenitor cell populations on bone formation. Biomaterials **31**: 1242-1250.

Lee CP, Colombo JS, Ayre WN, Sloan AJ, Waddington RJ (2015) Elucidating the cellular actions of demineralised dentine matrix extract on a clonal dental pulp stem cell population in orchestrating dental tissue repair. J Tissue Eng **6**: 1-13.

Lee K, Silva EA, Mooney DJ (2011) Growth factor delivery-based tissue engineering: general approaches and a review of recent developments. J R Soc Interface **55**: 153-170.

Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, Wozney JM, Kim EG, Choi JY, Ryoo HM, Bae SC (2000) Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol **20**: 8783-8792.

Lee MH, Kim YJ. Kim HJ, Park HD, Kang AR, Kyung HM, Sung JH, Wozney JM, Kim HJ, Ryoo HM (2003) BMP-2-induced Runx2 expression is mediated by Dlx5, and TGF-beta 1 opposes the BMP-2-induced osteoblast differentiation by suppression of Dlx5 expression. J Biol Chem **278**: 34387-34394.

Liu G, Xu G, Gao Z, Liu Z, Xu J, Wang J, Zhang C, Wang S (2016) Demineralized dentin matrix induces odontoblastic differentiation of dental pulp stem cells. Cells Tissues Organs **201**: 65-76.

Mafi P, Hindocha S,Mafi R, Griffin M, Khan WS (2011) Adult mesenchymal stem cells and cell surface characterization: a systematic review of the literature. Open Orthop J **5**, (Suppl 2-M4): 253-260.

Mansukhani A, Bellosta P, Sahni M, Basilico C (2000) Signaling by fibroblast growth factors (FGF) and fibroblast growth factor receptor 2 (FGFR2)-activating mutations blocks mineralization and induces apoptosis in osteoblasts. J Cell Biol **149**: 1297-1308.

Nastase MV, Young MF, Schaefer L (2012) Biglycan: a multivalent proteoglycan providing structure and signals. J Histochem Cytochem **60**: 963-975.

Nikitovic D, Aggelidakis J, Young MF, Iozzo RV, Karamanos NK, Tzanakakis GN (2012) The biology of small leucine-rich proteoglycans in bone pathophysiology. J Biol Chem **287**: 33926-33933.

Parisuthiman D, Mochida Y, Duarte WR, Yamauchi M (2005) Biglycan modulates osteoblast differentiation and matrix mineralization. J Bone Miner Res **20**: 1878-1886.

Park ES, Cho HS, Kwon TG, Jang SN, Lee SH, An CH, Shin HI, Kim JY, Cho JY (2009) Proteomics analysis of human dentin reveals distinct protein expression profiles. J Proteome Res **8**: 1338-1346.

Reis-Filho CR, Silva ER, Martins AB, Pessoa FF, Gomes PV, de Araújo MS, Miziara MN, Alves JB (2012). Demineralised human dentine matrix stimulates the expression of VEGF and accelerates the bone repair in tooth sockets of rats. Arch Oral Biol **57**: 469-476.

Roberts HC, Moseley R, Sloan AJ, Youde SJ, Waddington RJ (2008) Lipopolysaccharide alters decorin and biglycan synthesis in rat alveolar bone osteoblasts: consequences for bone repair during periodontal disease. Eur J Oral Sci **116**: 207-216.

Roberts TT, Rosenbaum AJ (2012) Bone grafts, bone substitutes and orthobiologics: the bridge between basic science and clinical advancements in fracture healing. Organogenesis **8**: 114-124.

Roberts-Clark DJ, Smith AJ (2000) Angiogenic growth factors in human dentine matrix. Arch Oral Biol **45**: 1013-1016.

Sadaghiani L, Gleeson HB, Youde SJ, Waddington RJ, Lynch CD, Sloan AJ (2016). Growth Factor

Liberation and DPSC Response Following Dentine Conditioning. J Dent Res **95**: 1298-1307.

Simmons CA, Alsberg E, Hsiong S, Kim WJ, Mooney DJ (2004) Dual growth factor delivery and controlled scaffold degradation enhance *in vivo* bone formation by transplanted bone marrow stromal cells. Bone **35**: 562-569.

Smith AJ, Cassidy N, Perry H, Bègue-Kirn C, Ruch JV, Lesot H (1995) Reactionary dentinogenesis. Int J Dev Biol **391**: 273-280

Smith AJ, Scheven BA, Takahashi Y, Ferracane JL, Shelton RM, Cooper PR (2012) Dentine as a bioactive extracellular matrix. Arch Oral Biol **57**: 109-121.

Tachi K, Takami M, Sato H, Mochizuki A, Zhao B, Miyamoto Y, Tsukasaki H, Inoue T, Shintani S, Koike T, Honda Y, Suzuki O, Baba K, Kamijo R (2011) Enhancement of bone morphogenetic protein-2-induced ectopic bone formation by transforming growth factor-β1. Tissue Eng Part A **17**: 597-606.

Waddington RJ, Hall RC, Embery G, Lloyd DM (2003a) Changing profiles of proteoglycans in the transition of predentine to dentine. Matrix Biol **22**: 153-161.

Waddington RJ, Roberts HC, Sugars RV, Schönherr E (2003b) Differential roles for small leucine-rich proteoglycans in bone formation. Eur Cell Mater **6**: 12-21.

Xu T, Bianco P, Fisher LW, Longenecker G, Smith E, Goldstein S, Bonadio J, Boskey A, Heegaard AM, Sommer B, Satomura K, Dominguez P, Zhao C, Kulkarni AB, Robey PG, Young MF (1998) Targeted disruption of the biglycan gene leads to an osteoporosis-like phenotype in mice. Nat Genet **20**: 78-82.

Young MF, Bi Y, Ameye L, Xu T, Wadhwa S, Heegaard A, Kilts T, Chen XD (2006) Small leucine-rich proteoglycans in the aging skeleton. J Musculoskelet Neuronal Interact **6**: 364-365.

Yu Y, Wang L, Yu J, Lei G, Yan M, Smith G, Cooper PR, Tang C, Zhang G, Smith AJ (2014) Dentin matrix proteins (DMPs) enhance differentiation of BMMSCs *via* ERK and P38 MAPK pathways. Cell Tissue Res **356**: 171-182.

Zara JN, Siu RK, Zhang X, Shen J, Ngo R, Lee M, Li W, Chiang M, Chung J, Kwak J, Wu BM, Ting K, Soo C (2011) High doses of bone morphogenetic protein 2 induce structurally abnormal bone and inflammation *in vivo*. Tissue Eng Part A **17**: 1389-1399.

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