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Biophysical and Biochemical Tools for Cell Phenotype Maintenance, Differentiation and Trans-Differentiation

A thesis submitted to the National University of Ireland Galway for the degree of Doctor of Philosophy

By

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Plagiarism Statement

I certify that this thesis is all my own work and I have not obtained a degree in this University or elsewhere on the basis of this work.

Diana Gaspar
### List of Abbreviations

- **α-SMA**: alpha smooth muscle actin  
- **µg**: microgram  
- **µm**: micrometre  
- **µM**: micromolar  
- **ACAN**: aggrecan  
- **ACTB**: beta actin  
- **ADF**: adult dermal fibroblasts  
- **ADSCs**: adipose derived stem cells  
- **ALPP**: alkaline phosphatase  
- **APS**: ammonium persulphate  
- **bFGF**: basic fibroblast growth factor  
- **BMP**: bone morphogenetic protein  
- **BMSCs**: bone marrow derived mesenchymal stem cells  
- **BSA**: bovine serum albumin  
- **CD**: cluster of differentiation  
- **C/EBPα**: CCAAT enhancer binding proteins  
- **COMP**: cartilage oligomeric matrix protein  
- **CR**: carrageenan  
- **CTGF**: connective tissue growth factor  
- **DAPI**: 4',6-diamidino-2-phenylindole  
- **DLS**: dynamic light scattering  
- **DMMB**: dimethylmethylene blue  
- **DMEM**: Dulbecco’s modified Eagle’s medium  
- **DNA**: deoxyribonucleic acid  
- **DTT**: dithiothreitol  
- **DxS**: dextran sulphate  
- **ECM**: extracellular matrix  
- **ESCs**: embryonic stem cells  
- **EVE**: excluded volume effect  
- **F**: Ficoll™ cocktail  
- **FBS**: foetal bovine serum  
- **Fc**: Ficoll™  
- **FGF**: fibroblast growth factor
FVO: fractional volume occupancy
GAGs: glycosaminoglycans
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GDF: growth differentiation factor
HAS: human serum albumin
HBSS: Hank’s balanced salt solution
HGF: hepatogenic growth factor
HIF: hypoxia inducible factor
HUVECs: human umbilical vein endothelial cells
Hz: Hertz
IBSP: integrin binding sialoprotein
IGF: insulin growth factor
IL: interleukin
IMR-90: human foetal lung fibroblast line
iPSCs: induced pluripotent stem cells
KSCs: kidney stem cells
M: molar
MDSCs: muscle derived stem cells
MMC: macromolecular crowding
MMP: metalloproteinase
NDF: neonatal dermal fibroblasts
nm: nanometre
Oct 4: octamer-binding transcription factor
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate buffered saline
PCL: Polycaprolactone
PCR: platelet clot releasate
PDGF: platelet derived growth factor
PDI: polydispersity index
PDMS: polydimethylsiloxane
PEG: polyethylene glycol
PFA: paraformaldehyde
PGA: polyglycolic acid
PLGA: poly(lactic-co-glycolic acid)
PLLA: poly(lactic acid)
PPARγ: peroxisome proliferator-activated receptor gamma
PRCR: platelet rich clot releasate
PRP: platelet rich plasma
PSCs: perivascular stem cells
PVP: polyvinylpyrrolidone
RIN: RNA integrity number
RIPA: radioimmunoprecipitation assay buffer
RNA: ribonucleic acid
rpm: revolutions per minute
RT: room temperature
RUNX2: runt-related transcription factor 2
SCXA: scleraxis
SDF1-α: stromal cell derived factor 1 alpha
SDS: sodium dodecyl sulphate
SPARC: secreted protein acidic and rich in cysteine
SSEA4: stage-specific embryonic antigen 4
TBS: Tris buffered saline
TEMED: tetramethylethylenediamine
TGF-β: transforming growth factor beta
THBS4: thrombospondin 4
TIMP: tissue inhibitor of metalloproteinases
TCs: Tenocytes
TNC: tenascin-C
TNMD: tenomodulin
TSCs: tendon stem cells
V: Volt
VEGF: vascular endothelial growth factor
WI-38: human lung fibroblast line
WS-1: human skin fibroblast line
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Abstract

Tendon injuries constitute an unmet clinical need, with 3 to 5 million new incidents occurring annually worldwide. Tissue grafting and biomaterial-based approaches fail to provide environments that are conducive to regeneration; instead they lead to nonspecific cell adhesion and scar tissue formation, which collectively impair functionality. Cell based therapies may potentially recover native tendon function, if tenocyte trans-differentiation can be evaded and cell differentiation / trans-differentiation towards tenogenic lineage is attained. To this end, recreating an artificial in vivo tendon niche by engineering functional in vitro microenvironments is a research priority. In this work, the effect of biophysical (macromolecular crowding, mechanical stimulation) and biochemical (oxygen tension) modulators on the behaviour of permanently differentiated cell sources (human adult and neonatal dermal fibroblasts and tenocytes) and stem cells (bone marrow derived mesenchymal stem cells) was assessed.

Firstly, the influence of hydrodynamic radius, charge and polydispersity of (a) various concentrations of different crowders (carrageenan, Ficoll™ and dextran sulphate); (b) various molecular weights of different crowders (70, 400 and 100 kDa of Ficoll™ and 10, 100 and 500 kDa of dextran sulphate) and (c) various cocktails of the same crowders (cocktails of various concentrations of different molecular weights Ficoll™ and dextran sulphate) on extracellular matrix deposition in human dermal fibroblast culture was analysed. The different crowders were tested individually and carrageenan was found to induce the highest extracellular matrix deposition due to its natural polydispersity and negative charge, conducive to more efficient volume exclusion. Cocktails of different molecular weight / concentrations of Ficoll™ or dextran sulphate, although presented increased polydispersity than their mono-domain counterparts, did not match carrageenan’s performance.

Carrageenan was utilised to accelerate extracellular matrix deposition in human bone marrow mesenchymal stem cells and it was found to outperform a Ficoll™ cocktail (70 kDa and 400 kDa). Additionally, under low oxygen conditions (2 %), increased matrix deposition was also observed. The multi-differentiation potential was maintained under both macromolecular crowding and hypoxia, though adipogenesis was significantly reduced after pre-treatment with 2 % oxygen tension.
and chondrogenesis was significantly increased after pre-conditioning with macromolecular crowding.

Finally, the combined effect of macromolecular crowding and mechanical stimulation on morphology, deposition of extracellular matrix and phenotype of human tenocytes, adult and neonatal dermal fibroblasts and bone marrow stem cells was assessed. Mechanical stimulation induced alignment perpendicular to the load of the permanently differentiated cell sources. Macromolecular crowding accelerated deposition of collagen type I. All cells deposited collagen types I, III, V and VI and bone marrow stem cells also deposited collagen type IV. Extracellular matrix synthesis was not increased by mechanical stimulation. Gene expression analysis revealed upregulation of scleraxis by tenocytes, potentially indicating phenotypic maintenance. Both neonatal and adult dermal fibroblasts presented upregulation of thrombospondin 4 and alkaline phosphatase or cartilage oligomeric protein, respectively, whilst the expression profile of bone marrow stem cells was unchanged.

Taken together, these results provide further knowledge on the use of biophysical and biochemical in vitro microenvironment modulators for control of cell phenotype and development of tissue-like supramolecular assemblies.
Chapter 1 - Introduction

Sections of this chapter have been published in:


¹Shared first authorship
1.1 Introduction

Over 30 million musculoskeletal injuries occur annually worldwide and nearly half of them involve tendon and ligament injuries. The US and EU associated expenditure exceeds US $180 billion annually. With the increase in life expectancy, it is predicted that tendon injuries will continue to rise, placing an enormous financial strain on healthcare systems [1]. As tendon healing is slow and leads to fibrotic scarring and adhesions, the natural repair process is not sufficient to functionally repair the injured tissue [2-8]. Current strategies to manage mild tendon injuries resort to conservative treatments (e.g. rest, physiotherapy and pharmacological methods) of questionable efficiency [9-14]. In severe injuries, tissue grafts remain the gold standard in clinical practice. However, autograft-induced site morbidity should be minimal and should result in less disability than the original injury; these prerequisites limit the availability of suitable autologous tissues. Allografts and xenografts, although are more readily available than autografts and have demonstrated proportional clinical outcomes, are associated with a different set of concerns, including inadequate processing that may jeopardise mechanical properties; possibility of disease transmission; and immune mediated rejection [15-18]. These limitations have triggered an intense investigation into tissue engineering and biomaterial-based alternatives to tissue grafts. To-date, numerous two- and three-dimensional; nano- to macro-; and bottom-up to top-down fabrication technologies (e.g. self-assembly [19], electrospinning [20], freeze drying [21], imprinting [22]) and synthetic [e.g. poly(ε-caprolactone), PCL [23]; poly(glycolic acid), PGA [24]; poly(lactic acid), PLLA [25]; poly(lactic-co-glycolic acid), PLGA [26]] or natural (e.g. collagen type I [27], silk [28]) in origin biomaterials alone or in combination with bioactive/therapeutic molecules [e.g. glycosaminoglycans (GAGs) [29], growth factors [30], genes [31]] have been investigated \textit{in vitro} and \textit{in vivo}. Despite the very promising preliminary results, so far, these constructs have not completely recapitulated native tendon composition, structure/architecture and mechanical properties. In fact, very frequently, such approaches have been associated with inflammation, hypercellularity, calcification, and inadequate mechanical properties and tissue organisation, imposing the need for new functional tendon therapies and highlighting the need for new cell-based approaches inspired in the physiological environment [32-37].
1.2 Extracellular and cellular composition of tendon

Tendons are responsible for two major physiological roles: structural integrity and transmission of mechanical forces from muscle to bones [2]. Tendons are composed of dense connective tissue consisting of a highly organised hierarchical structure of collagen interspersed with cells and a network of other non-collagenous molecules (Figure 1.1) [38]. Tendons are composed of 50-70% water and collagen constitutes around 60-80% of their dry weight, with collagen type I being the predominant type [39]. During development, tendon formation starts with nucleation of small collagen fibril intermediates which assemble lengthwise to form long collagen chains, which in turn fuse laterally to form the thicker fibres that constitute the mature tissue [40]. Tendon is encapsulated by a thin sheet of connective tissue known as epitenon which contains fascicles aligned parallel to the axis of mechanical loading [41]. Fascicles are covered by another layer of connective tissue denominated endotenon which facilitates fascicle gliding. Epitenon and endotenon also provide vascular, lymphatic and nerve supply [40].

Although type I collagen is the most abundant, other types of collagen can be found in tendon. Other forms of fibrous collagens such as types III and V can be found in fibrils in smaller quantities and their presence can influence the fibril growth during development and mechanical properties of the tissue [42, 43]. These fibrillar types of collagen consist of long triple helical structures composed of three polypeptide chains that form quarter staggered fibrils. Collagen I is a heteropolymer composed of two $\alpha_1$(I) chains and one $\alpha_2$(I) chain while collagen III consists of three identical $\alpha_1$(III) chains and collagen V can consist of three $\alpha_1$(V), two $\alpha_1$(V) and one $\alpha_2$(V) or $\alpha_1$(V), $\alpha_2$(V) and $\alpha_3$(V) [44]. Collagen types VII and IX also have regulatory roles in tendon development [45]. Non-collagenous components of tendon extracellular matrix (ECM) include elastin and proteoglycans. The elastin content in a tissue relates to its elasticity and in tendon it constitutes around 1 - 2 % of the dry mass being responsible for the elastic behaviour of tendon (recovery after stretching) [38, 46]. Proteoglycans are glycoproteins that consist of a core protein attached to GAG chains. There are two major groups of proteoglycans that can be distinguished depending on their molecular weight: the small leucine rich proteoglycans (<100 kDa), which include decorin, biglycan, lumican, fibromodulin, and cartilage oligomeric matrix protein (COMP) and can interact with other ECM proteins such as collagen or fibronectin and can also bind to growth
factors including transforming growth factor beta (TGF-β) and epidermal growth factor (EGF); and large or modular proteoglycans (>1000 kDa), such as aggrecan and versican [47, 48]. Overall, proteoglycans compose less than 1 % of the dry mass of tendon, but they can influence several important processes such as collagen fibrillogenesis, cell-cell interactions and growth factor binding [49]. Decorin, fibromodulin and lumican bind to the surface of collagen fibrils and during embryonic development, can modulate fibril formation, lateral growth and size [47, 50]. GAGs are linear carbohydrates that can be divided in chondroitin sulphate, dermatan sulphate, keratin sulphate, heparin and hyaluronan [47]. In foetal tendinous tissue, chondroitin sulphate and hyaluronan are the dominant types, while in adult tissue zone dermatan sulphate is present in higher amounts as part of decorin and biglycan [51]. Dermatan sulphate can bind to collagen type I and it is involved in regulation of ECM assembly [52].

The ECM components of tendons are secreted by tenocytes and tenoblasts which are, respectively, elongated fibroblast-like cells that are aligned parallel to the collagen fibrils, and round cells with an ovoid nuclei [38, 53]. These cells compose most of the cellular population in tendon, nonetheless other cells that have been identified include chondrocytes at the osteotendinous junction and synovial, endothelial and smooth muscle cells in the tendon sheath and vasculature [54-56]. Although the development of tendon and the phenotype of the different cell types are not well characterised, it is known that tenoblasts are more abundant in younger tendons and more active in terms of matrix production and remodelling [57, 58]. During the ageing process, these cells mature into tenocytes and become more elongated and display a decrease in intracellular organelles; but interconversion between the two phenotypes also appears to be related with response to external stimuli such as exercise or trauma [58, 59].
Figure 1.1: Bottom-up assembly of the tendon unit. Collagen type I exists as an heterotrimer of two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain. (A) Collagen type I is synthesised intracellularly in its precursor form, procollagen, with intact $N$- and $C$-terminal propeptide extensions. (B) Following or during secretion into the extracellular space, specific propeptide cleavage takes place via the respective $N$- and $C$-proteinases. (C) Assembly of collagen molecules into quarter-staggered fibrils. A native cross-linking pathway will promote staggered assembly of collagen fibrils in a head-to-tail fashion. (D) Collagen fibrils will then form collagen fibres, which will then be joined together to form bundles and, ultimately, the tendon unit.
1.3 Phenotypic drift and alternative cell sources for tendon repair

Although there have been significant developments in cell therapies for tendon regeneration, there are still many issues to be addressed in terms of the choice of cell source and optimal culture conditions for phenotype maintenance, when tenocytes are used, or tenogenic differentiation, when undifferentiated cells are involved. Tenocyte culture remains problematic, with serial passaging resulting in reduced metabolic activity and tenogenic marker losses (e.g. collagen type I, tenomodulin, tenascin-C and scleraxis) [60-62]. Given their limited availability and low activity, it is imperative to obtain an efficient expansion method that will preserve their phenotype and function in vitro. To this end, the use of three-dimensional high-density cultures was advocated, given that such systems closely imitate the native environment of the host tissue, maintain physiological tenocyte morphology and increase, albeit in a transient fashion, tenogenic marker expression (e.g. scleraxis) [26, 63, 64]. However, a similar transient increase in cartilage oligomeric matrix protein (COMP) and aggrecan expression may indicate trans-differentiation towards chondrogenic lineage [26]. These data along with the need for high cell number, often not available, to maintain a high-density culture have compromised extensive investigation in this area.

Although efforts are being directed towards overcoming issues with current protocols for culture of tenocytes, alternative cell sources are also under investigation. To-date, cell populations that have been assessed for tendon repair can be clustered as: permanently differentiated cells (e.g. tenocytes, dermal fibroblasts, muscle cells); undifferentiated/progenitor/stem cell types [e.g. bone marrow stem cells (BMSCs), adipose-derived stem cells (ADSCs), tendon stem cells (TSCs), perivascular stem cells (PSCs), muscle-derived stem cells (MDSCs), and embryonic stem cells (ESCs)]; and reprogrammed / engineered cells [e.g. induced pluripotent stem cells (iPSCs), reprogrammed / engineered / genetically modified cells induced to upregulate the expression / production of a specific molecule] (Table 1.1).

The most studied cell types for tendon repair are tenocytes and BMSCs. Tenocytes are the most obvious choice for cell-based therapies for tendon repair as they are the native cell population but their culture is still problematic. BMSCs have also been preferred due to their differentiation potential and regenerative potential in their undifferentiated state.
Similarly to tenocytes, dermal fibroblasts have spindle shape morphology and ECM secretome (e.g. collagen types I and III, fibronectin, decorin) [65, 66] and unlike tenocytes, they grow well in culture and are readily available. For these reasons, their potential in tendon regeneration is under investigation. However, tenogenic markers are yet to be assessed in detail and it is unclear to what extent tenogenic differentiation of dermal fibroblasts can be achieved.

Akin to tenocytes, skeletal muscle-derived cells are exposed to high mechanical loads; express collagen types I, III and VI, scleraxis, tenomodulin, and growth differentiation factor 8 (GDF-8); and unlike tenocytes, they grow well in culture [67-69].

The discovery and detailed characterisation of TSCs with the capacity to form a tendon-like tissue in vitro and in vivo [70] meant that we may now have at our disposal a tissue-specific stem cell population. This notion is further supported by experimental data demonstrating superior BMSC clonogenicity and proliferation capacity; higher octamer-binding transcription factor (Oct) 4 expression; and much higher expression of tenomodulin, scleraxis, collagen type I, decorin, biglycan and fibromodulin [71]. Given however their limited availability (3 to 4 % of the total tendon cell population) [72] and their readiness to lose their phenotype in culture with time and passaging [73], it is imperative to develop means to maintain their phenotype and function or to promote their tenogenic differentiation.

Given that the intra-patellar fat pad contains high number of ADSCs, it has been postulated that ADSCs may play a significant role in the healing of injured patellar tendons [74]. Further, ADSCs proliferate faster than tenocytes, sheath fibroblasts and BMSCs; have similar in vitro growth characteristics to the aforementioned cells; and implantation studies in a rabbit flexor profundus tendon defect showed similar histological characteristics for all these cell types [75]. These data clearly indicate the potential of ADSCs for tendon repair and regeneration. As with BMSCs, the concern for potential ossification has triggered investigation into their tenogenic induction using various stimuli.

Other stem cell sources have also been applied to tendon regeneration. Perivascular stem cells have been identified in different tissues/organs [76], including tendon [77, 78]. Naïve, equine in origin, umbilical cord blood mesenchymal stem cells have shown promising results in various equine injuries and pathophysiologies [79,
Further, equine blood-derived stem cells stimulated by macrophage colony-stimulating factor resulted in the successful repair of equine tendon injuries [81]. Despite the safety concerns associated with ESCs, their potential in tissue engineering and regenerative medicine is still under intense investigation [82-85]. Although the beneficial potential of naïve ESCs has been shown in equine models [86, 87], there is still no work in clinical setting. It is also important to mention the potential of genetically modified cells that can be altered to express molecules of interest [31, 88-94] (Table 1.1). However, results to date have not yet fully corroborated the potential of this approach, particularly when there are safety concerns regarding the gene delivery vehicles used. Overall, it is still not clear which is the optimal cell population for tendon regeneration due to the lack of standardised protocols and readout systems and further in-depth studies are required.
Table 1.1: Rationale for each cell population currently employed in tendon repair and regeneration strategies.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Cell Population</th>
<th>Rationale / Advantages</th>
<th>Concerns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permanently differentiated cells</td>
<td>Tenocytes</td>
<td>The predominant cell type in tendon, responsible for producing the extracellular milieu of the tendon tissues.</td>
<td>Limited availability of functional tenocytes.</td>
</tr>
<tr>
<td></td>
<td>Dermal fibroblasts</td>
<td>Readily available in large numbers; grow well <em>in vitro</em>; have spindle-shaped morphology and similar protein secretome as tenocytes.</td>
<td>Specificity issues.</td>
</tr>
<tr>
<td>Muscle cells</td>
<td></td>
<td>Readily available in large numbers; grow well <em>in vitro</em>; share some markers with tenocytes.</td>
<td>Specificity issues.</td>
</tr>
<tr>
<td>Stem Cells</td>
<td>BMSCs</td>
<td>Readily available; very well characterised; can differentiate towards tenogenic lineage; secrete an array of regenerative factors.</td>
<td>In undifferentiated state, may lead to ectopic bone formation; developmental stage mismatch, when differentiated towards tenogenic lineage.</td>
</tr>
<tr>
<td></td>
<td>ADSCs</td>
<td>Readily available; fairly well characterised; can differentiate towards tenogenic lineage; secrete an array of regenerative factors.</td>
<td>In undifferentiated state, may lead to ectopic bone formation; developmental stage mismatch, when differentiated towards tenogenic lineage.</td>
</tr>
<tr>
<td><strong>TSCs</strong></td>
<td>Tendon-specific stem cells; higher proliferation capacity than BMSCs; express tendon-specific markers.</td>
<td>Limited availability; in undifferentiated state, may lead to ectopic bone formation; developmental stage mismatch, when differentiated towards tenogenic lineage.</td>
<td></td>
</tr>
<tr>
<td><strong>MDSCs</strong></td>
<td>Readily available in large numbers; can differentiate towards tenogenic lineage; secrete an array of regenerative factors.</td>
<td>In undifferentiated state, may lead to ectopic bone formation; developmental stage mismatch, when differentiated towards tenogenic lineage.</td>
<td></td>
</tr>
<tr>
<td><strong>PSCs</strong></td>
<td>They share the same marker expression with tenocytes and BMSCs.</td>
<td>In undifferentiated state, may lead to ectopic bone formation; developmental stage mismatch, when differentiated towards tenogenic lineage.</td>
<td></td>
</tr>
<tr>
<td><strong>ESCs</strong></td>
<td>They can recapitulate developmental stages of tendon tissues; can differentiate towards tenogenic lineage.</td>
<td>Tumour induction; ethical issues; in undifferentiated state, may lead to ectopic bone formation; developmental stage mismatch, when differentiated towards tenogenic lineage.</td>
<td></td>
</tr>
<tr>
<td>Engineered / Reprogrammed cells</td>
<td>iPSCs</td>
<td>Pluripotent; engineered from readily available adult cells.</td>
<td></td>
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<tr>
<td>--------------------------------</td>
<td>-------</td>
<td>----------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>↑ <strong>Scleraxis</strong></td>
<td>High expression in tendon progenitor cells.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑ <strong>Smad8</strong></td>
<td>Involved in tenogenic differentiation.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑ <strong>BMP-2</strong></td>
<td>Enhances healing in the tendon-bone junction.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑ <strong>VEGF</strong></td>
<td>Induces angiogenesis; promotes vasculogenesis, stimulates cell migration; inhibits apoptosis.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑ <strong>IGF-1</strong></td>
<td>Enhances tendon healing and tenocyte proliferation.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑ <strong>GDF-5</strong></td>
<td>Induces neotendon formation and promotes tendon healing.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑ <strong>PDGF</strong></td>
<td>Enhances tendon healing and tenocyte proliferation.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑ <strong>FGF-2</strong></td>
<td>Promotes collagen production, tendon development and tenocyte proliferation.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 1

1.4 Biophysical, biochemical and biological tools for \textit{in vitro} modulation of tendon microenvironment

In recent years, various \textit{in vitro} microenvironment modulators in the form of biophysical cues, biochemical switches and biological signals [95] are under intense investigation, aiming to create an \textit{in vitro} niche similar to the native tendon from which the cells were derived from. Among them, substrate architecture, media supplementation with bioactive molecules, mechanical stimulation and oxygen tension have been extensively studied as means to control tenocyte phenotype and function \textit{ex vivo}. Although macromolecular crowding [96, 97] and substrate elasticity [98, 99] have being recognised as key modulators of the \textit{in vitro} microenvironment, the influence of these stimuli on tenocyte phenotype and function is still under-studied.

1.4.1 Substrate composition and architecture

One of the more recurrent approaches for tenogenic phenotype maintenance and differentiation is the use of patterned substrates that mimic the structural and topographical features of the physiological environment [100-102]. To imitate the intricate network of aligned collagen fibres, aligned micro-fibres and micro-grooves have been used and have been shown to promote elongated cell morphology, collagen and proteoglycan production and tenomodulin expression [103-105]. Moreover, after culture on smooth surfaces, marker expression has been shown to be restored by culture on micro-grooves [104]. However, nano-sized features have been described to be unsuitable in promoting collagen and tenomodulin expression [106], which indicates that structures in the micron range have a more relevant role in maintaining tenogenic phenotype as this better mimics the diameter of the collagen fibres found in tendon (1-4 $\mu$m).

Using decellularised tendon slices human and rat dermal fibroblasts maintained their spindle shape morphology only for 6 h, whilst on oriented collagen type I multi-lamellar membranes, human dermal fibroblasts maintained their spindle shape morphology for up to 12 days in culture [65]. Gene analysis studies demonstrated that foetal fibroblasts expressed higher levels of collagen type I, collagen type III and fibromodulin than their adult counterparts, making them a better choice for tendon tissue engineering [107]. Further, in a three-dimensional
environment, rat foetal dermal fibroblasts demonstrated better attachment, alignment and infiltration into the scaffold than their adult counterparts [108]. When loaded on a PGA electro-spun construct and subjected to mechanical loading, human dermal fibroblasts formed in vitro a mature, highly aligned ‘tendon-like’ tissue [109]. When muscle-derived cells were seeded on decellularised tendon matrix, they exhibited similar time to confluency and collagen and proteoglycan synthesis as tendon-derived cells and superior to bone marrow-derived cells [110]. Multifactorial approaches based on aligned sub-micron fibres loaded with FGF-2 promoted parallel to the fibre orientation cell alignment and tenogenic induction, whilst inhibited myocyte differentiation of mouse C2C12 myoblasts and primary MDSCs; although bone morphogenetic protein 2 (BMP-2) upregulated scleraxis, the simultaneous increase of alkaline phosphatase indicated differentiation towards osteogenic lineage [111, 112]. As with dermal fibroblasts, in the absence of any other studies, it is unclear to what extent tenogenic differentiation of muscle derived cells / MDSCs can be achieved.

Tendon-derived matrix has also been shown to stimulate TSC proliferation and to preserve more effectively their stemness and/or tenogenic phenotype in comparison to naked tissue culture plastic [113] and bone- or skin-derived matrix [114].Aligned electro-spun PLLA fibres have been shown to promote spindle-shaped cell morphology, to significantly upregulate tendon specific genes (e.g. collagen type I, collagen type III, collagen type XIV, scleraxis, elastin) and to downregulate osteogenic genes (e.g. osteocalcin, alkaline phosphatase, RUNX2), even in osteogenic media [115].

The tenogenic differentiation potential of BMSCs has been assessed using various natural and synthetic polymers and scaffold conformations, including electro-spun silk [116-118] and poly(lactic-co-glycolic acid) (PLGA) [119] anisotropic mats; imprinted polydimethylsiloxane (PDMS) substrates [22]; collagen [120] and fibrin [121, 122] hydrogels; collagen sponges [123, 124]; electrochemically aligned collagen fibres [39]; decellularised allogeneic tendons [75] and cryopreserved tendons [125]. Among them, anisotropically aligned devices are considered to be more effective, as they recapitulate native tendon architecture; promote physiological elongated nuclei/cytoskeleton morphology; enable matrix deposition parallel to the substrate orientation; and upregulate tendon markers (e.g. scleraxis,
tenomodulin), whilst suppressing osteogenic markers (e.g. osteocalcin); and ultimately trigger differentiation towards fibroblastic/tenogenic lineage [39, 126]. ADSCs can maintain collagen type I expression when loaded on a collagen gel and injected into a tendon graft, but COMP expression is also increased which may indicate chondrogenic differentiation [127]. When equine ADSCs were cultured in Matrigel™ in the presence and absence of FGF2 and FGF5; an increased scleraxis and tenascin-C expression was observed on Matrigel™ seeded cells, with no notable difference in the expression of these markers when supplementation with FGF2 or FGF5 took place [128]. Human ADSCs on naïve PCL electro-spun scaffolds and scaffolds coated with tendon-derived matrix or fibronectin resulted in increased sulphated GAG content; increased gene expression of collagen type I, decorin and tenascin-C; and increased mechanical properties, with no notable difference among the groups [129]. A collagen/tendon-derived matrix scaffold loaded with ADSCs and subjected to uniaxial tension significantly increased cell proliferation; reduced expression activity of metalloproteinases (MMPs); and induced tenogenic differentiation [130]. Three-dimensional culture on electro-spun PLGA scaffolds supplemented with GDF5 significantly upregulated gene expression of collagen type I and scleraxis, as opposed to two-dimensional PLGA films [131]. Aligned collagen fibres loaded with nano-particles carrying PDGF induced tenogenic differentiation of ADSCs, as evidenced by increased expression of tenomodulin and scleraxis and suppressed osteogenic induction, as evidenced by unchanged levels of alkaline phosphatase and osteocalcin [132].

Furthermore, culture of equine ESCs in a three-dimensional collagen scaffold upregulated expression of collagen type I and tenascin-C; the subsequent addition of TGF-β3 brought about an increase in tenomodulin and thrombospondin 4, indicative of tenogenic differentiation [133]. Although no areas of bone or cartilage were observed, upregulation of COMP may be of concern, especially in long-term culture. A more recent study demonstrated that force alone induced osteogenic differentiation of human ESCs, whilst in combination with scleraxis induced tenogenic differentiation [134].

1.4.2 Media supplementation

The beneficial effects of animal [135] and human [136, 137] sera have long been recognised as means to promote in vitro cell growth. Specifically to tenocyte
culture, foetal bovine serum (FBS) is traditionally used to maintain their growth in culture [138]. However, serum supplementation has been shown to have a detrimental effect in tenocyte culture, with the cells readily trans-differentiating towards an osteoblast-like lineage, increasing expression of alkaline phosphatase and osteopontin and decreasing decorin and tenomodulin expression [139]. To this end, serum supplements (e.g. ascorbate, insulin / transferrin / selenium cocktail, growth factors) have been used to either completely replace animal/human sera or to compensate-suppress the adverse effects of serum use. Ascorbate plays an important role in collagen synthesis [140, 141] and has been shown to increase collagen production in tenocyte culture [142-145]. However, the actual influence of ascorbate on the expression of tenogenic markers has yet to be assessed. In vitro supplementation with insulin/transferrin/selenium supports cell proliferation / growth and prevents apoptosis, even at low or no serum supplementation, as insulin promotes glucose uptake, intracellular transport, nucleic acid/protein synthesis; transferrin, an iron chelator, reduces toxic levels of oxygen radicals and peroxide; and selenium, a trace element and a co-factor of various proteins, has an antioxidant effect [146-150]. Although insulin / transferrin / selenium cocktail has been used in tenocyte culture in the absence or in the presence of low concentration of FBS and/or growth factors [30], its effect as a stand-alone supplement on tenogenic marker expression is still to be assessed. Given that growth factors (e.g. insulin-like growth factor, IGF; platelet derived growth factor, PDGF; basic fibroblast growth factor, bFGF; transforming growth factor β, TGF-β) and cytokines (e.g. BMPs; interleukin, IL) are playing a pivotal role in the tendon healing process [151-153], numerous studies have assessed their influence on tenocyte culture in two- and three-dimensional settings, with variable degree of efficiency. For example, bFGF was found to enhance proliferation and upregulate collagen type I expression in rabbit tenocytes [154]. However, using a heparin-binding delivery system, although bFGF has been shown to promote proliferation and to upregulate decorin lubricant and hyaluronic acid synthase 2 gene expression after 10 days in canine tenocyte culture, it induced downregulation of collagen type I and collagen type III after 10 days in culture, whilst MMP-1 and MMP-13 were significantly up-regulated [155]. PDGF-BB has been shown to promote proliferation and to upregulate decorin lubricant and hyaluronic acid synthase 2 gene expression after 10 days in canine tenocyte culture. Although the overall collagen content was increased, gene

15
analysis indicated unaffected expression of MMP-1 and MMP-13, upregulation of collagen type III and downregulation of collagen type I [155, 156]. Another study demonstrated that PDGF-BB and bFGF supplementation increased canine flexor tendon cell proliferation and collagen production, whilst VEGF and BMP-2 did not affect cell proliferation and collagen production [157]. BMP-12 in human patellar tendon tenocytes has been shown to increase cell proliferation and gene expression of collagen type I and collagen type III; however the observed decrease in decorin expression questions its efficacy [158]. BMP-13 in human patellar tendon tenocytes increased proliferation and the expression of collagen type I [159]; although IL-4 and IL-13 have been shown to increase proliferation of human tenocytes, none of them increased collagen expression [160]. In a three-dimensional setting, induced by an anisotropic collagen-GAG porous scaffold, PDGF-BB and IGF-1 enhanced equine tenocyte motility, viability and metabolic activity [29]. In a follow-up and far more detailed study, where the influence of stromal cell-derived factor 1α (SDF1α), PDGF-BB, IGF-1, GDF-5 and bFGF was assessed on equine tenocytes seeded on an anisotropic collagen-GAG porous scaffold, it was found that PDGF-BB and IGF-1 maintain tenocyte proliferation, whilst GDF-5 and bFGF maintain phenotype, as assessed by gene expression of collagen markers (e.g. collagen type I, collagen type III, tenascin-C, decorin, scleraxis) and COMP [161]. IL-1β has been shown to upregulate elastin and MMP expression and to downregulate collagen type I expression on human tenocytes in a bioartificial tendon [162]. When equine superficial digital flexor tendon explants treated with IGF-1, an increase in collagen synthesis and cell proliferation was noted [163], whilst human recombinant PDGF-BB supplementation resulted in increased collagen type I gene expression, decreased collagen type III gene expression and unchanged GAG content [164]. However, it has now become apparent that sequential or simultaneous supplementation of multiple growth factors is more beneficial than a single growth factor / singular supplementation. Indeed, the sequential use of combinations: PDGF / bFGF (14 days expansion phase) and TGF-β3 / IGF-1 (14 days differentiation phase) has been shown to promote proliferation and collagen type I production respectively [165]. Simultaneous supplementation of PDGF-BB / bFGF in canine flexor tendon cell culture increased proliferation, as compared to individual growth factor supplementation [157]. When equine tenocytes were seeded on an collagen-GAG aligned scaffold and subsequently subjected to growth
factor cocktails, it was found that the IGF-1 / GDF-5 and IGF-1 / bFGF were more effective with respect to tenocyte phenotype, gene expression and proliferation, as compared to PDGF-BB / bFGF and PDGF-BB / GDF-5 [161]. TGF-β / IGF-1 mixture has been shown to increase expression of scleraxis, tenomodulin and decorin [30, 166]; IGF-1 / PDGF-BB / bFGF has been shown to significantly enhance cell proliferation [167]; BMP-2 / BMP-7 cocktail has been shown to stimulate cell proliferation, collagen type I and collagen type III expressions and collagen type I synthesis in both young and old subjects [168]. An alternative strategy to literally infinite growth factor supplementation schemes, with respect to dose, growth factor choice and time of supplementation, is based on platelet rich plasma (PRP) supplementation, due to its inherent high growth factor and platelet content [169-171]. Data to-date demonstrate that PRP supplementation increases cell proliferation; stimulates vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) production [172]; improves ECM organisation [173]; and inhibits myofibroblastic differentiation [174]. However lack of detailed compositional analysis and batch-to-batch variability makes comparison between experimental data impossible.

Similarly to tenocytes, growth factor and cytokine supplementation have been at the forefront as means to maintain TSC phenotype, with variable results to-date. IGF-1 supplementation preserved TSC multipotency for up to 28 days, whilst upregulated decorin and scleraxis expression and GDF-5 treatment promoted differentiation towards tenocytes [175]. Culture of TSCs with BMP-2 and TGF-β1 inhibited the expression of scleraxis, tenomodulin and SOX9, and did not affect the expression of levels of COMP [176]. BMP-2 induced osteogenic, adipogenic and chondrogenic differentiation of TSCs and inhibited tenogenic marker expression (e.g. decorin, biglycan, fibromodulin) [177]. The addition of prostaglandin E2 in TSC culture decreased cell proliferation and increased BMP2 production, which subsequently induced osteogenic differentiation [178]. Further, it has been shown that TSCs exhibit higher osteogenic differentiation with / without BMP-2 stimulation as compared to BMSCs [179]. TGF-β1 induced differentiation towards fibrocartilage or calcified tendon lineage [175]. Platelet rich clot releasate (PRCR) supplementation encouraged TSCs to assume an elongated morphology, enhanced cell proliferation and collagen production and promoted tenogenic (e.g. collagen type I, collagen type III, tenascin-C), rather than adipogenic (e.g. PPARγ),
chondrogenic (e.g. SOX9) or osteogenic (e.g. RUNX2) gene expression [115]; however an observed increase in \(\alpha\)-SMA may indicate trans-differentiation towards myofibroblastic lineage. When rat patellar tendon TSCs were treated with connective tissue growth factor (CTGF) and ascorbic acid, a significant increase in mRNA expression of tendon specific markers (e.g. tenomodulin, scleraxis, thrombospondin 4, collagen type I, elastin, decorin, biglycan) and a significant decrease in chondrogenic (e.g. collagen type II, aggrecan) and osteogenic (e.g. osteocalcin) markers was noted [180].

*In vitro* studies have also shown that ascorbate and GDF-5 supplementation increased tenomodulin, collagen type I and collagen type III expressions on skeletal MDSCs and exhibited a more organised appearance than BMSCs [181].

The potential of growth factor, hormone or cytokine supplementation, on tenogenic differentiation of BMSCs has also been extensively assessed with variable degree of efficiency. GDF-5 increased total collagen expression, significantly upregulated scleraxis, tenasin-C and collagen type I and significantly downregulated runt-related transcription factor 2 (RUNX2) and sex determining region Y-box 9 (SOX9) [182]; GDF-6 significantly increased mRNA expression of tenomodulin and scleraxis and induced anisotropic cell orientation [183]; bFGF enhanced mRNA expression of collagen types I and III and fibronectin, whilst homogeneous, dense, fibroblast-like, spindle-shaped cells with long cell processes were evidences; however, an increased mRNA expression of \(\alpha\)-SMA may indicate trans-differentiation towards myofibroblastic lineage [184]; insulin enhanced expression of collagen types I and III, scleraxis, tenasin-C and decorin [185]; BMP-12 upregulated expression of scleraxis and tenomodulin [186]. BMSC treated with CTGF and ascorbic acid increased the synthesis of collagen type I and tenasin-C, without remarkable change in cell morphology, as compared to the control counterparts [187]. More complex culture environments have also been assessed, once more, with variable degree of efficacy. An increased tenogenic commitment of human BMSCs was evidenced by increased expression of collagen type I, collagen type III, scleraxis and tenomodulin, when they were cultured under the synergistic effect of aligned chitosan-PCL nano-fibres and TGB-\(\beta\)3 [23]. Co-culture of BMSCs with tenocytes resulted in upregulated gene expression of collagen type I, collagen type III, scleraxis and tenasin-C [188, 189]. IGF-1 / TGF-\(\beta\)1 cocktail increased expression of scleraxis and tenomodulin; and PDGF-BB
resulted in moderate increases in collagen type I, collagen type III, scleraxis, tenomodulin and decorin expression [188]. Porous PCL scaffold coated with collagen and GDF-5 and subsequently subjected to static or cyclic strain (10% strain, 0.33 Hz) increased cellular metabolism and mRNA production of collagen type I and scleraxis, whilst tenascin-C production remained unaffected [190]; however the observed increase in mRNA of collagen type II may be indicative of chondrogenic differentiation. Although bFGF stimulated expression of collagen type I, collagen type III, fibronectin and tenasin-C [184, 191], a concurrent increase of α-SMA may indicate trans-differentiation towards myofibroblastic lineage.

Simple systems based on GDF5 supplementation have also been applied to ADSCs and were shown to increase cell proliferation; protein expression of tenomodulin, tenasin-C, Smad-8 and MMP13; to enhance gene expression of scleraxis, tenomodulin and tenasin-C; and to increase production of collagen type I and decorin [192]. However a parallel increase in aggrecan production may be indicative of trans-differentiation towards chondrogenic lineage. More complex multifactorial systems have also been assessed with variable degree of effectiveness. Although insert system and tenocyte conditioned media increased tenasin-C expression, direct co-culture with tenocytes induced the highest proliferation and tenasin-C expression [193].

Moreover, BMP12 supplementation of PSCs can induce tenogenic differentiation, as evidenced by increased expression of collagen type I, scleraxis, tenomodulin and decorin [194]. Further, when equine umbilical cord blood stem cells were cultured in Matrigel™ in the presence of FGF5, proliferation was stimulated and scleraxis expression was increased [128]. Although mesenchymal stromal cells derived from peripheral blood enhanced tendon healing in a sheep deep digital flexor tendon model, addition of PRP did not induce any additional effect [195].

Regarding ESCs, TGF-β3 supplementation of equine cells resulted in upregulation of tendon associated protein and genes (e.g. collagen type I, scleraxis, tenasin-C, tenomodulin), suggesting successful in vitro differentiation towards tenogenic lineage [196], however thrombospondin-4 was not detected and COMP was also upregulated, which is indicative of chondrogenic trans-differentiation.
1.4.3 Mechanical stimulation

Bearing in mind that the main function of tendon is to transfer mechanical loads from muscle to bone, mechanical stimulation is another very important factor to consider, when emulating *in vitro* a functional tenogenic microenvironment. Constant strains applied to avian tenocytes on aligned electro-spun PGA fibres led to thin and highly compacted collagen fibres, suggesting that constant strain may not be the optimal means of mechanical load [24]. On a PCL scaffold, rabbit Achilles tendon tenocytes showed enhanced proliferation and collagen deposition under dynamic loading, as compared to static culture counterparts [197]. Further studies have demonstrated that combinations of cyclic loading, increasing strain levels and rest periods are important parameters in maintaining mouse tail tenocyte phenotype, as assessed by scleraxis and collagen type I gene expression [198], rabbit flexor digitorum profundus tendon tenocyte phenotype, as evidenced by increased cell proliferation, collagen type I synthesis and maintenance of physiological cell morphology [199] and rat tail tenocytes, as indicated by increase in hyaluronic acid and cluster of differentiation (CD) 44 [200]. Further, amplitude and frequency of cyclic strain has been shown to modulate MMP and tissue inhibitor of metalloproteinase (TIMP) expression in tenocytes from various species and tissues [201-203]. Several studies have also demonstrated differential regulation of protein and gene expression in tenocytes, as a function of applied load regime, on isolated tendon fascicles from various species [204-210]. In a proteomics analysis study of human tenocytes loaded on aligned PGA scaffolds and subjected to dynamic loading revealed that, when compared the loaded to the unloaded counterparts, 195 proteins were significantly upregulated and 189 proteins were significantly downregulated [211], which not only indicates the complexity of mechanotransduction in cellular function, but also clearly demonstrates the need for sophisticated readout systems to appreciate induced changes.

Mechanical loading has also been shown to influence rabbit patellar and Achilles tendon TSCs differentiation in a stretching magnitude-dependent manner. Specifically, mechanical loading of 4 % promoted tenogenic differentiation, whilst 8 % stretching directed TSCs towards adipogenic, chondrogenic, and osteogenic lineages [212]. In another study, both 4 % and 8 % stretching induced upregulation of BMP2, which subsequently increased the osteogenic differentiation of rat
patellar TSCs, as evidenced by increased alkaline phosphate activity and calcium nodule formation [213]. A more recent study demonstrated that even 2% stretching for 3 days is capable of inducing osteogenic differentiation of rat patellar TSCs, as evidenced by increased expression of RUNX2 and alkaline phosphatase activity [214]. Nonetheless, the addition of PRCR on TSCs subjected to 8% mechanical stretching increased the number of cells and the concentration of collagen type I, collagen type III and TGF-β1, whilst significantly reduced expression of peroxisome proliferator-activated receptor γ (PPARγ), SOX9 and RUNX2 and adipogenic, chondrogenic and osteogenic differentiated cells [215]. This notable difference in lineage commitment may be attributed to the different tissues/species from which the cells were extracted from, once more indicating that standardised methodologies, reporting and readout systems should be established.

Mechanical stimulation has also been proposed as an optimal in vitro niche for BMSCs differentiation towards tenogenic lineage. Mechanical stimulation (10% stretching, 1 Hz frequency) increased the expression of collagen type I, collagen type III and tenascin-C in rat BMSCs [216]. Collagen-based materials loaded with BMSCs and subsequently mechanically stimulated (2.4% stretching, 0.0033 Hz frequency) yielded a significantly stronger tendon-like tissue than their non-stimulated counterparts [123, 217]. Mechanical stimulation (5 to 10% stretching, 0.1 to 1 Hz frequency) of BMSCs combined with three-dimensional collagen-based hydrogels [218], aligned fibrous scaffolds [118] or GDF-5 loaded porous PCL scaffolds [190] has been shown to increase expression of tendon markers (e.g. collagen type I, scleraxis, tenascin-C and tenomodulin).

Mechanical stimulation (10% strain, 1 Hz frequency) of ESCs loaded on a silk-collagen composite scaffold exhibited tenocyte-like morphology and expressed collagen type I, collagen type III and scleraxis in vitro, ectopic implantation in nude mice resulted in a tendon-like tissue with better alignment and protein expression than constructs without any mechanical stimulation and when implanted in injured murine tendons, they outperformed their counterparts [28]. Stepwise differentiation of human ESCs (via plating, ascorbic acid supplementation and static mechanical loading) improved regeneration in rat patellar tendons [219].

Considering the multiplicity of mechanical loading conditions and setups in place, it is still not clear which are the optimal conditions for tenogenic differentiation of the various cell populations.
1.4.4 Oxygen tension

Considering the avascular nature of tendon tissue, and thus its low oxygen tension, culture under low oxygen tension conditions has been postulated to create an optimal *in vitro* niche for tenocyte phenotype maintenance. So far, it has been shown that low oxygen tension (2 %) increases the expansion capability of porcine tenocytes, without altering the expression of ECM components (e.g. collagen I, collagen III, decorin) [220]. However, culture of human tenocytes at 0.1 % oxygen tension resulted in increased production of pro-inflammatory cytokines, altered matrix regulation and increased expression of apoptotic mediators [221, 222], suggesting that such low oxygen tension conditions can be recruited for the development of pathophysiological models. The synergistic effect of 5% oxygen tension and PDGF supplementation resulted in 5-fold increase of VEGF in rat Achilles tendon tenocytes [223], a molecule with significant effect on MMP and TIMP expression and tendon pathophysiology and angiogenesis [224]. Although these conflicting results may be attributed to the oxygen tension difference, the origin of the cells (e.g. species, tissue and tissue function) cannot be excluded, imposing once more the need for a standardised experimental setup and readout system.

Given the low vascularity of tendon tissue, low oxygen tension has been hypothesised to create an optimal *in vitro* niche for TSCs phenotype maintenance. However, low oxygen tension (2 %) culture of human patellar tendon TSCs did not increase metabolic rate; increased cell number; and reduced multilineage potential, with associated increase in lineage specific markers such as osteocalcin and alkaline phosphatase (osteogenic lineage); C/EBPα and PPARγ2 (adipogenic lineage); aggrecan and SOX9 (chondrogenic lineage); and tenomodulin (tenogenic lineage) [225], indicating no particular effect towards tenogenesis. However, a more recent study assessing the influence of 5 % oxygen tension on human TSCs demonstrated maintenance of stemness, as evidenced by upregulation of Nanog, Oct4, nucleostemin and SSEA4 [226].

1.4.5 Macromolecular crowding

One underappreciated yet essential parameter to consider when recreating the physiological environment *in vitro* is the density of macromolecules surrounding
the intra- and extra- cellular environment. In tissues, the presence of different macromolecules creates a confined or crowded environment that has a significant effect on the rate of biological reactions and chemical equilibria [227, 228]. This is referred to as ‘crowded’ as opposed to ‘concentrated’ as it is created by a variety of macromolecules without any single molecule occurring at high concentration. The molecular crowding is due to the excluded volume effect created by non-specific repulsion interactions between the different macromolecules that depends on their size, shape and charge [229]. The in vitro setting typically used in standard cell culture protocols represents a much more dilute environment (total molecule concentration of 1-10 mg/ml) [230], which causes significant decreases in the rate of biological processes.

The effects of macromolecular crowding have been studied in different systems, such as cells and bacteria [97, 231], with applications including cell shape and function [232, 233], enzymatic activity and reaction equilibria [229, 230], protein structure, folding and assembly [234-238] and DNA condensation [239, 240]. Another biological process significantly affected by these diluted conditions is ECM deposition. This has been shown to affect particularly molecules which are dependent on enzymatic processes. This is the case of collagen, the dominant structural molecule in many tissues and organs but it is not seen, for example, with fibronectin, as the latter is deposited through a cell-mediated process [241, 242]. To create a more confined setting in vitro, inert macromolecules can be added to the culture medium. In the specific case of collagen deposition this has been shown to increase the rate of deposition, by accelerating cleavage of N- and C- propeptides of the procollagen molecule by proteases, thereby significantly reducing culture time needed to create tissue-like constructs in vitro.

Several different macromolecules have been used to enhance ECM deposition (detailed list can be found in Table 1.2), including neutral and negatively charged with a wide molecular weight range. However, it has been seen that the size, charge and polydispersity of the crowder have a significant effect on the modulation of the excluded volume effect and therefore on the deposition of ECM and potentially on cell behaviour [96, 243]. More specifically, higher molecule polydispersity has been described to further accelerate ECM deposition [96] but it appears that the choice of crowder can also affect cell phenotype as seen in corneal fibroblasts, where myofibroblastic differentiation can occur when dextran sulphate is used
[244], or with BMSCs, where adipogenesis appears to be enhanced when a Ficoll™ cocktail was utilised [245]. Polydispersity refers to the heterogeneity of sizes of molecules in a mixture and it contributes for maximising the excluded volume effect, as the larger particles exclude most of the volume but smaller ones can still fill in empty spaces available, further increasing the confinement in the environment. This prompted the use of mixed crowding, where artificial polydispersity can be created by mixing different molecules or different molecular weights of the same molecule. Interestingly, the use of a mixture of Ficoll™ and dextran has been reported through to have a greater than additive effect on protein stabilisation [246, 247]. However, there is still limited experimental data on mixed crowding. The most common example is the Ficoll™ 70 and 400 cocktail. This has been used to enhance ECM deposition in human corneal fibroblasts [248], WI-38 fibroblasts [249] and porcine chondrocytes [250]. The fabrication of rich in ECM decellularised BMSCs matrices has also been enhanced by adding dextran sulphate 10 kDa to the Ficoll™ cocktail [251]. Both dextran 70 kDa and Ficoll™ 70 kDa have been added to bovine serum albumin to analyse the effects of mixed MMC on protein folding [252]. Nonetheless, further studies are required to further understand the effects of mixed crowding and better choose the adequate crowder for the application and cell type required.
### Table 1.2: Macromolecular crowders used *in vitro* for cell culture.

<table>
<thead>
<tr>
<th>Macromolecular Crowder</th>
<th>Cell type</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Hexamethylene bisacetamide | Human malignant mesenchymal cell line | Increased procollagen synthesis  
Morphological changes | [253] |
| PEG 4 kDa  
Dextran T40  
Polyvinylpyrrolidone | Human dermal fibroblasts | Full processing of procollagen to collagen | [254] |
| Dextran sulphate 500 kDa | Human dermal fibroblasts | Full proteolytic processing of procollagen to collagen | [255] |
| Dextran T40 | Human dermal fibroblasts | Increased deposition of collagen III | [256] |
| Dextran sulphate 500 kDa | Mutated dermal fibroblasts | Increased fibronectin  
Presence of mutant collagen trimers affected ECM composition | [257] |
| Dextran 670 kDa  
Dextran sulphate 500 kDa | WI-38 | Increased ECM deposition | [258] |
| Dextran sulphate 10 kDa and 500 kDa  
Polysodium-4-styrene 200 kDa  
Ficoll™ 70 kDa  
Ficoll™ 400 kDa | WI-38 | Accelerated conversion of procollagen to collagen  
Crowder size and charge affect deposition | [259] |
| Dextran sulphate 500 kDa  
Ficoll™ 70 kDa  
Ficoll™ 400 kDa | IMR-90  
WI-38 | Increased ECM deposition  
Establishment of fibrosis model | [249] |
<table>
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<th>Crowder</th>
<th>Cell Type</th>
<th>Phenotype Response</th>
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<tr>
<td>Dextran sulphate 500 kDa</td>
<td>WI-38</td>
<td>Increased ECM deposition&lt;br&gt;Stable propagation of embryonic stem cells</td>
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<td>Ficoll™ 70 kDa&lt;br&gt;Ficoll™ 400 kDa</td>
<td>Porcine chondrocytes</td>
<td>Increased deposition of total collagens and glycosaminoglycans</td>
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<td>Human BMSCs</td>
<td>Increased deposition of collagen I and ECM alignment</td>
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<td>Human BMSCs</td>
<td>Increased deposition of collagen IV and perlecan, enhanced adipogenesis</td>
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<td>WI-38&lt;br&gt;WS-1&lt;br&gt;Human tenocytes&lt;br&gt;Human osteoblasts</td>
<td>Increased ECM deposition modulated by crowder polydispersity</td>
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<td>Human corneal fibroblasts</td>
<td>Increased ECM deposition&lt;br&gt;Phenotype maintenance</td>
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<td>Dextran sulphate 500 kDa&lt;br&gt;Carrageenan</td>
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<td>Increased ECM deposition&lt;br&gt;PVP360 increases cell proliferation</td>
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<td>MMC can be used to derive proangiogenic cells with therapeutic efficacy for treatment of limb ischemia</td>
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<td>Vocal fold fibroblasts</td>
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<td>Carrageenan</td>
<td>Human corneal fibroblasts</td>
<td>MMC combined with 2% oxygen tension enhances ECM synthesis and deposition</td>
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<td>Human ADSCs</td>
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<td>Ficoll™ 400 kDa</td>
<td>HUVECS Murine kidney stem cells (KSCs)</td>
<td>MMC enhanced reconstitution of kidney derived matrices that supported morphogenesis of HUVECS and KSCs</td>
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1.5 Preclinical and clinical assessment

Cell-mediated tendon engineering therapies are promising alternatives to traditional graft / scaffold treatments, given the low activity / low cell number of tendons. In cell-mediated repair, cell suspensions can be injected at the site of injury or implanted in the form of cell sheets or along with a tissue graft or a scaffolding material to enable homogeneous cell distribution and localisation at the side of injury [1, 72, 217, 271-290]. As the interest in cell-based therapies for tendon repair grows, it is becoming apparent that the most important aspect is the choice of cell population. Although numerous studies have assessed the influence of in vitro microenvironment modulators on tenocyte phenotype and function, only a handful has assessed the clinical relevance of the derived cells in preclinical and clinical settings. Ex vivo TGF-β supplementation of subsequently implanted tenocytes improved the biomechanics of the regenerated rabbit patellar tendon tissue [291], whilst ex vivo PRP treatment produced more collagen fibres than non-stimulated cells in a diffusion chamber in a rat chest cavity model [292]. Ex vivo mechanical stimulation of tenocytes led to increased elastic modulus and ultimate strength of the regenerated tissue [293]. At the moment, there are two clinical trials in progress (source: ClinicalTrials.gov, Trial Number: NCT01343836 and source: ClinicalTrialsRegister.eu, Trial Number: 2010-021869-73) investigating the therapeutic potential of autologous tenocytes in chronic tendinopathy. Results from a clinical trial that assessed the influence of direct injection of autologous tenocyte for chronic lateral epicondylitis demonstrated reduction in pain, significant improvement of grip strength 12 months post-injection and reduction in tear size [245]. Unfortunately, all preclinical and clinical studies lack in depth biological analysis. Extrapolating from the positive in vitro results, a few studies have assessed the influence of direct growth factor injections, hormone injections, cytokine injections, PRP injections or post-injury exercise on the properties of the recovering tendon, once more, with variable degree of efficiency. Local IGF-1 injections enhanced protein synthesis within and around human patellar tendons [294, 295]. Local injections of circulating growth hormone increased collagen synthesis in human patellar tendons [296]. IL-6 increased collagen synthesis in the peritendinous tissue of human Achilles tendons on both resting subjects and individuals assigned to an exercise regime [297]. With respect to PRP, variable and often contradictory results are reported from clinical trials; hence long-term studies,
with proper control groups and thorough analysis are essential to ensure safety and clinical effectiveness [226, 298-300]. With respect to mechanical loading, acute exercise has been shown to increase formation of collagen type I in peritendinous Achilles tendon tissue in humans [301-303] and to even improve the clinical outcome of human patellar tendinopathy, most likely due to the production of new collagen [304].

In a mouse Achilles tendon model, foetal fibroblasts promoted a better healing than their adult counterparts, as evidenced by much better structural and functional properties; higher levels of collagen deposition; recruitment of fibroblast-like cells at the site of injury; and reduction in the number of infiltrated inflammatory cells at the site of injury [107]. Using a porcine flexor digital superficial tendon defect model, the clinical potential of autologous tenocytes and dermal fibroblasts loaded on an electro-spun PGA scaffold was assessed. At 14 and 26 weeks post-implantation both tenocyte and dermal fibroblast-based substitution exhibited similar histological and biomechanical properties of about 75% of natural tendon strength [305]. However, no further marker analysis was carried out to assess whether the dermal fibroblasts had been trans-differentiated into functional tenocytes. Despite the very limited in vitro and preclinical assessment work of dermal fibroblasts for tendon repair, three clinical studies have already been completed. The first one reported, using autologous cells, decrease in pain, in the number of recurrent tears, in the number of new vessels and in tendon thickness in patients with lateral epicondylitis [306]. The second one reported that patients with patellar tendinopathy improved faster, when treated with autologous dermal fibroblasts; only one patient suffered a rupture and required supplementary surgery [307]. The third study demonstrated the safety of dermal fibroblast injections for the treatment of refractory Achilles tendinosis [308]. Again, it is worth pointing out that no studies have assessed in preclinical or clinical setting the influence on differentiated towards tenogenic lineage dermal fibroblasts.

In a subcutaneous mouse model, skeletal muscle-derived cells loaded on PGA fibres produced a thicker and mechanically stronger neotissue than the one produced by their tenocyte counterparts. Interestingly, 24 weeks post-implantation, muscle cells ceased expression of desmin and increased tenomodulin expression, suggesting that this cell source could be useful for tendon repair [67]. No other
preclinical / clinical investigations are available using muscle derived cells or MDSCs. Implantation studies of whole bone marrow cells or purified BMSCs have resulted in ectopic bone formation in rat [238], mouse [239] and rabbit [240–242] models, independently of the scaffold chemistry (e.g. collagen, PGA) and architecture (e.g. hydrogels, sponges). Thus, *in vitro* tenogenic induction may be necessary, although such approach would jeopardise the bioactivity of stem cells. A collagen sponge loaded with BMP-12 treated rat BMSCs formed a tendon-like tissue in a rat calcaneal tendon defect, as evidenced by increased cell number, elongation and alignment along the tensile axis, greater matrix deposition and elevated expression of tendon markers (e.g. collagen type I, scleraxis, tenomodulin and tenascin-C) [186]. Further a small intestine submucosa (SIS) scaffold loaded with GDF-6 treated BMSCs promoted tendon regeneration in nude mice and in a rat patellar tendon window injury model [183]. Mechanically stimulated BMSCs on a collagen sponge improved material properties, biomechanics and tendon marker expression (e.g. collagen type III and decorin) in a rabbit patellar tendon model [123, 124, 309]. Similarly, hypoxic preconditioning has been postulated to improve functional repair, given that BMSCs cultured at 1 % O\textsubscript{2} increased collagen type I and collagen type III production and improved the biomechanics of the neotissue in a rat Achilles tendon model [310]. However, it is worth pointing that the observed increase in collagen synthesis under low oxygen culture is more likely to be due to the fact that HIF-1\textalpha increases the transcription of prolyl-4-hydroxylase, which is involved in collagen synthesis [311, 312]. Several studies have assessed the influence of BMSCs in equine patients [313-316], with data to-date demonstrating increased collagen type I production; improved histological scores; and reduction in cellularity and vascularity. There are also two clinical trials in progress investigating the efficacy of BMSCs in the treatment of refractory Achilles tendinopathy (source: Australian New Zealand Clinical Trials Registry, Trial Number: ACTRN12610000985088) and rotator cuff tear repair (source: ClinicalTrials.gov, Trial Number: NCT01687777). In both human and equine patients, naïve BMSCs were used.

As with BMSCs, several studies advocate the regenerative potential of naïve ADSCs in small and large animal preclinical models [288, 317-323], but only one study has assessed the reparative capacity of pre-treated ADSCs. Specifically,
ADSCs seeded on electro-spun PLGA scaffolds and subsequently subjected to dynamic stretch, when implanted in a rabbit Achilles tendon model exhibited histological structure similar to that of native tendon [324]. With respect to clinical investigations of ADSCs for tendon repair, only one study has been reported using autologous ‘fat grafting’ to treat a young athlete with persistent patellar tendon tears. However, bone marrow aspirate was added to the lipoaspirate and therefore the treatment could have been due to the combinatory effect of both ADSCs and BMSCs. However, given that the ADSC fraction was several hundred times of the BMSCs, the study has been reported as an ADSC success story [325]. There is one clinical trial in preparation that proposes to investigate the effect of injecting allogeneic ADSCs for treating lateral epicondylitis (source: ClinicalTrials.gov, Trial Number: NCT01856140).
1.6 Project rationale and hypotheses

Cell therapies for tendon repair have seen significant advances in the last decade. However, there is an urgent need for standardised testing of outcomes to accurately assess the suitability of the different cell populations and *in vitro* differentiation tools for tendon regeneration. Furthermore, given the complexity of the native tendon environment and most recent research efforts, the use of multifactorial approaches for cell phenotype maintenance and differentiation is required. As such, the use of biochemical and biophysical tools to modulate cell phenotype and ECM deposition using different cell sources relevant for tendon repair and regeneration is herein proposed. To attain this goal, the following phases and hypotheses were established:

**Phase 1:** Modulation of *in vitro* microenvironment for enhanced ECM deposition.
**Hypothesis:** Deposition of extracellular matrix by human dermal fibroblasts can be modulated by the biophysical properties (molecular weight, charge, hydrodynamic radius, polydispersity) of different crowders.

**Phase 2:** Macromolecular crowding and low oxygen tension for human mesenchymal stem cell phenotype maintenance and enhanced ECM deposition.
**Hypothesis:** The combined use of macromolecular crowding and low oxygen tension will enhance ECM deposition, whilst maintaining the bone marrow mesenchymal stem cells’ undifferentiated phenotype.

**Phase 3:** Tenogenic phenotype maintenance and trans-differentiation / differentiation using macromolecular crowding and mechanical stimulation.
**Hypothesis:** A multifactorial approach based on mechanical loading and macromolecular crowding can maintain tenocyte phenotype, differentiation bone marrow stem cells towards tenogenic lineage and trans-differentiate neonatal and adult dermal fibroblasts towards tenogenic lineage.
1.7 References


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213. Rui, Y.F., P.P.Y. Lui, M. Ni, L.S. Chan, Y.W. Lee, and K.M. Chan, Mechanical loading increased BMP-2 expression which promoted osteogenic


Chapter 1


Chapter 1


Chapter 2 - Modulation of \textit{in vitro} microenvironment for enhanced extracellular matrix deposition

\textbf{Sections of this chapter have been submitted for publication:}

Polydispersity is key modulator of extracellular matrix deposition under macromolecular crowding conditions. \textbf{D. Gaspar}, K. P. Fuller, D. I. Zeugolis, Submitted.
2.1 Introduction

Direct cell injections or cell-assembled tissue-engineered devices require extended *in vitro* culture to either attain suitable cell numbers [1, 2] or sufficient ECM deposition [3-5], respectively. However, the more the cells reside in the artificial and far from physiological *in vitro* culture microenvironment, the more they lose their phenotype, function and therapeutic potential [6, 7]. In the last decade, substantial work has been conducted in recreating physiological [8, 9] and pathophysiological niches [10, 11] *in vitro* for regenerative medicine and drug discovery purposes. Media supplements [12, 13], oxygen tension [14, 15], 2D / 3D scaffolds with precise architectural [16-18] and biomechanical features [19] and bioreactor systems capable of applying various forces [20, 21] have been used extensively with variable degree of efficiency, considering the literally infinite number of permutations (e.g. concentrations, combinations and timings of media additives; architectural and biomechanical features of the scaffolds; mechanical loading regimes). The influence of light [22, 23] and gravity [24, 25] has also been assessed, but to a lesser extent. It is striking though that although cells live in a highly dense extracellular space, the influence of localised density has been overlooked with only a handful of studies having assessed the influence of either overlay [26, 27] or macromolecular crowding (MMC) [28, 29] (more efficient than overlay as more effectively excludes volume) in cell culture systems.

MMC is a biophysical phenomenon based on the volume excluded by macromolecules present in an environment that occurs naturally in the native intra- and extra- cellular space [30]. This derives from molecules being mutually impenetrable, therefore excluding the volume they occupy to other molecules, which is referred to as the excluded volume effect (EVE) [31]. The extent of MMC depends on the shape, size, charge and concentration of the crowder. Theoretical and computational models have significantly contributed in understanding this phenomenon and how it affects biological reactions [32, 33]. However, they are not coming without limitations. For example, different models have been used to describe the dependence of radius on concentration for the same molecule (e.g. dextran has been modelled as having its radius independent [34], linearly [35] or quadratically [36] dependent of concentration [37]); they do not account for the full range of inter- and intra- molecular interactions due to high computational / processing power requirements [38]; and they do not directly correlate with
experimental data [37]. This poses the need for more experimental work to fully comprehend the potential of macromolecular crowding.

Early experimental work demonstrated that MMC affects DNA condensation [39], protein interactions [40], the rate of enzymatic reactions [41] and cell function [42]. In cell culture settings, at first instance, MMC was shown to increase collagen type I deposition by enhancing the activity of procollagen C- and N- proteinases [29, 43]. Since then, numerous studies have demonstrated the positive impact of different crowders [carrageenan (CR) [44, 45], Ficoll™ (Fc) [46, 47], dextran sulphate (DxS) [48], polysodium-4-styrene sulphonate (PSS) [49], polyvinylpyrrolidone (PVP) [50]] in permanently differentiated and stem cell culture. Among the various macromolecules that have been used to-date, CR [44] and DxS [48] appear to be the most efficient in excluding volume, due to their inherent polydispersity. A Fc cocktail (70 and 400 kDa) has also shown promise [46, 47, 49], as crowding cocktails, by creating an artificial polydispersity, can more effectively exclude volume than their mono-domain counterparts [44, 51]. Yet again, no study has assessed in a systematic fashion the influence of crowder(s’) concentration, molecular weight, charge, hydrodynamic radius and polydispersity in a cell culture system. Herein, we assessed the influence of various concentrations of CR, Fc (70, 400 and 1,000 kDa alone or in cocktail) and DxS (10, 100 and 500 kDa alone or in cocktail) in ECM deposition of human primary adult dermal fibroblasts.
2.2 Material and methods

2.2.1 Materials
Tissue culture consumables were purchased from Sarstedt (Ireland) and NUNC (Denmark). All chemicals, cell culture media and reagents were purchased from Sigma Aldrich (Ireland), unless otherwise stated.

2.2.2 Dynamic light scattering (DLS) measurements
Hydrodynamic radius, polydispersity (PDI) and zeta potential of different concentrations of CR, DxS (10 kDa, 100 kDa and 500 kDa alone or in cocktail; TdB Consultancy, Sweden) and Fc (70 kDa, 400 kDa, and 1,000 kDa alone or in cocktail; TdB Consultancy, Sweden) were assessed using dynamic light scattering (Zetasizer ZS90, Malvern Instruments, UK). Range of concentrations of Fc 1,000 to be used was determined by a linear regression using the previously published optimal concentrations of Fc 70 and 400. The crowding solutions were prepared in Hank’s Balanced Salt Solution (HBSS) to mimic physiological conditions. Fractional volume occupancy (FVO) was calculated using the obtained values of hydrodynamic radius for the different crowders, as previously published [28] and the best-fit trend line was applied to the data.

2.2.3 Cell culture experimentation
Human adult dermal fibroblasts (American Type Culture Collection, UK), used between passages 2 and 5, were expanded in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 1 % penicillin streptomycin and 10 % foetal bovine serum at 37 ºC in a humidified atmosphere of 5 % CO₂. For MMC experiments, cells were seeded at 25,000 cells / cm² density in 24 well plates and were allowed to attach for 24 h. Subsequently, the media were changed with media containing 100 µM of L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate and CR, Fc 70, 400 and 1,000 kDa (alone or in cocktail) or DxS 10, 100 and 500 kDa (alone or in cocktail). ECM deposition was assessed after 3, 7 and 14 days in culture. The mediaf were changed every 3 days.
2.2.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)
At the various time points (3, 7 and 14 days), culture media were aspirated and cell layers were briefly washed with HBSS. Cell layers were then digested with pepsin from porcine gastric mucosa (Sigma Aldrich, Ireland) at 0.1 mg / ml in 0.5 M acetic acid (Fischer Scientific, Ireland) at 37 °C for 2 hours under agitation. Cell layers were then scraped and neutralised with 1 M sodium hydroxide (Sigma, Ireland). Cell layer samples were analysed by SDS-PAGE under non-reducing conditions using a Mini-Protean 3 system (Bio-Rad Laboratories, UK). Bovine collagen type I (100 µg / ml, Symatese Biomateriaux, France) was used as standard for all gels. Staining of the protein bands was performed with SilverQuest™ kit (Thermo Fisher Scientific, UK) following manufacturer’s instructions.

2.2.5 Phase contrast microscopy
Phase contrast images were captured using an inverted microscope (Leica Microsystem, Germany) at different time points (3, 7, 14 days) to evaluate the influence of different MMC conditions on cell morphology. Images were processed using ImageJ software (NIH, USA).

2.2.6 Cell viability
At the various time points (3, 7 and 14 days), calcein AM (Thermo Fisher Scientific, UK) and ethidium homodimer I (Thermo Fisher Scientific, UK) stainings were performed, as per manufacturer’s protocol, to assess the influence of the different crowders on cell viability. Briefly, cells were washed with HBSS and a solution of calcein AM (4 µM) and ethidium homodimer I (2 µM) was added. Cells were incubated at 37 ºC and 5 % CO₂ for 30 minutes after which, fluorescence images were obtained with an Olympus IX-81 inverted fluorescence microscope (Olympus Corporation, Japan).

2.2.7 Cell metabolic activity
At the different time points (3, 7 and 14 days), the alamarBlue® assay (Thermo Fisher Scientific, UK) was used to evaluate the influence of MMC on cell metabolic activity, as per manufacturer’s instructions. Briefly, at each time point, cells were washed with HBSS and a 10 % alamarBlue® solution in HBSS was added to the
cells. Cells were incubated at 37 ºC and 5 % CO₂ for 3 hours and absorbance was measured at 550 nm and 595 nm with a Varioskan Flash Spectral scanning multimode reader (Thermo Fisher Scientific, UK). Cell metabolic activity was expressed as percentage reduction of the alamarBlue® dye and normalised to the non-crowded control (-MMC).

2.2.8 Immunocytochemistry
At each time point (3, 7 and 14 days), cells were briefly washed with HBSS and fixed with 2 % paraformaldehyde for 15 minutes at room temperature. Cells were washed again and non-specific site interactions were blocked with 3 % bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 30 min. Cells were incubated for 90 minutes at room temperature with the primary antibody (mouse anti-collagen type I, rabbit anti-collagen type III; Abcam, UK), after which, they were washed 3 times with PBS, followed by 30 minutes of incubation at room temperature with the secondary antibody (AlexaFluor® 488 goat anti mouse, AlexaFluor® 488 chicken anti rabbit; Thermo Fisher Scientific, UK). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescent images were captured with an inverted fluorescence microscope (Olympus IX-81, Olympus Corporation, Tokyo, Japan) and images were processed with ImageJ software (NIH, USA).

2.2.9 Statistical analysis
Data are expressed as mean ± standard deviation. Statistical analysis was performed using MINITAB® version 17 (Minitab Inc., USA). One-way analysis of variance (ANOVA) was used for multiple comparisons and Tukey’s post hoc test was used for pairwise comparisons after confirming the samples followed a normal distribution (Kolmogorov-Smirnov test) and had equal variances (Bartlett’s and Levene’s test for homogeneity of variances). When either or both assumptions were violated, non-parametric analysis was conducted using Kruskall-Wallis test for multiple comparisons and Mann-Whitney test for pairwise comparisons. Statistical significance was accepted at $p<0.05$. 
2.3 Results

2.3.1 Carrageenan (CR)

Zeta potential measurements showed that the charge of CR was negative and significantly \(p<0.001\) decreased with increasing its concentration (Figure 2.1A). The hydrodynamic radius (Figure 2.1B) and polydispersity (Figure 2.1C) also significantly \(p<0.05\) increased with increasing its concentration. An exponential increase of fractional volume occupancy (FVO) was observed with increasing concentrations of CR (Figure 2.2). SDS-PAGE (Figure 2.3A) and complementary densitometric analysis of collagen \(\alpha_1(1)\) bands (Figure 2.3B) demonstrated that the presence of CR increased collagen type I deposition in a concentration-dependent manner and that the 100 and 500 \(\mu g/ml\) induced significantly \(p<0.001\) higher deposition (almost 5-fold increase over the non-crowded control). No statistical significance \(p>0.05\) was found between 100 and 500 \(\mu g/ml\), suggesting that 100 \(\mu g/ml\) was sufficient to achieve maximum ECM deposition. Concentrations above 500 \(\mu g/ml\) were not used, as they altered the viscosity of the culture medium. At day 7 and 14, collagen type V deposition was also evident (Figure 2.3A). Immunocytochemistry analysis for collagen type I confirmed the concentration-dependent increase in collagen type I deposition (Figure 2.4A) and a similar trend was observed for collagen type III (Figure 2.4B). Cell morphology (Figure 2.5A), viability (Figure 2.5B) and metabolic activity (Figure 2.5C) were not affected as a function of CR concentration.
Figure 2.1: DLS measurements of various CR concentrations. (A) Zeta potential decreases significantly with increasing concentrations of CR ($p<0.001$). (B) Hydrodynamic radius and (C) PDI increased significantly with increasing concentrations of CR ($p<0.05$). Measurements at 10 $\mu$g/ml were not attainable as they were below the detection limit of the equipment.
Figure 2.2: Fractional volume occupancy (FVO) for different concentrations of carrageenan (A), Fc 70 (B), Fc 400 (C), Fc 1,000 (D) and Dxs 500 (E).
Figure 2.3: Collagen I deposition at various concentrations of CR. (A) SDS-PAGE for collagen type I deposited by adult dermal fibroblasts at 3, 7 and 14 days of culture with increasing concentrations of CR (10, 50, 100 and 500 µg/ml) and a non-crowded control (-MMC). At day 14, 100 µg/ml shows higher collagen type I deposition when compared with all other groups. (B) Densitometric analysis of collagen α1(I) bands of SDS-PAGE gels representing collagen I deposition in the presence of various concentrations of carrageenan and a non-crowded control (-MMC) at days 3, 7 and 14.
Figure 2.4: Immunocytochemistry images showing deposition of collagen type I (A) and collagen type III (B) at 3, 7 and 14 days using different concentrations of carrageenan.
Figure 2.5: Cellular morphology (A), viability (B) and metabolic activity (C) of adult dermal fibroblasts at days 3, 7 and 14 with varying concentrations of carrageenan. Cells maintained their spindle shaped morphology throughout the time points and metabolic activity was not significantly affected. Viability was also unaffected (green cells: live; dead cells: red).
2.3.2 Ficoll™ (Fc)

For the various concentrations / molecular weight Fc molecules, the zeta potential (Figure 2.6A, Figure 2.6D and Figure 2.6G) was found to either be neutral or to have a nominal negative charge (above -5 mV). Fc 70 (Figure 2.6A) presented significantly higher ($p<0.001$) charge at 37.5 mg/ml, whilst Fc 400 (Figure 2.6D) presented significantly lower ($p<0.001$) charge at 100 mg/ml. Fc 1,000 (Figure 2.6G) did not exhibit significant changes with concentration ($p>0.05$). The hydrodynamic radius of Fc 70 (Figure 2.6B) and 1,000 (Figure 2.6H) did not vary significantly with concentration ($p>0.05$), whilst, the radius of Fc 400 (Figure 2.6E) was found to decrease with increasing concentrations ($p<0.05$). Polydispersity of Fc 70 (Figure 2.6C) and Fc 400 (Figure 2.6F) increased significantly ($p<0.01$ and $p<0.001$, respectively) with concentration, whilst increased concentrations of Fc 1,000 did not significantly ($p>0.05$) affect polydispersity (Figure 2.6I). FVO of Fc 70 was observed to increase linearly with increasing concentration, reaching a maximum of 60 % at 100 mg/ml (Figure 2.2B). As for Fc 400, FVO increased linearly with concentration, stabilising at approximately 25 % when concentration was higher than 25 mg/ml (Figure 2.2C). FVO of Fc 1,000 presented a similar trend to Fc 70, also exhibiting a linear increase with concentration and achieving a maximum of above 40 % at 10 mg/ml (Figure 2.2D). SDS-PAGE (Figure 2.7A) and complementary densitometric analysis of collagen $\alpha_{1}(I)$ bands (Figure 2.8A) showed that Fc 70, at day 14, induced the highest ($p<0.001$) collagen deposition at 37.5 mg/ml. Fc 400 presented the highest ($p<0.001$) collagen type I deposition at day 14 at 25 mg/ml, as revealed by SDS-PAGE (Figure 2.7B) and complementary densitometric analysis (Figure 2.8B). Fc 1,000 did not exhibit collagen I deposition at any time point (Figure 2.7C). Contradictory to the SDS-PAGE results, immunocytochemistry analysis revealed that no collagen I had been deposited at either time point when Fc 70 was used (Figure 2.9A). Deposition of collagen type III was found to increase in the presence of Fc 70, particularly at day 3 at concentrations above 37.5 mg/ml, though it was also found in the -MMC groups at days 7 and 14 (Figure 2.9B). Similarly, immunocytochemistry analysis also demonstrated that increasing concentrations of Fc 400 did not lead to presence of collagen type I in the cell layer (Figure 2.10A), whilst collagen type III was increased at 50 and 100 mg/ml, although at a lower
extent than observed with Fc 70 (Figure 2.10B). As for Fc 1,000, immunostaining analysis revealed absence of collagen type I, which was concordant with SDS-PAGE findings (Figure 2.11A), and, although some collagen type III was found, the use of increasing concentrations of Fc 1,000 did not induce an increase over the -MMC counterparts (Figure 2.11B). Cell morphology (A), viability (B; p>0.05) and metabolic activity (C; p>0.05) were not affected by the various concentrations of different molecular weight Fc 70 (Figure 2.12), Fc 400 (Figure 2.13) and Fc 1,000 (Figure 2.14).
Figure 2.6: DLS measurement of different molecular weight Fc molecules (70, 400 and 1,000 kDa) at different concentrations. (A) Zeta potential of Fc 70 was significantly increased at 37.5 mg/ml ($p<0.001$). (B) Hydrodynamic radius did not vary significantly with
concentration. (C) Polydispersity increased significantly with increasing concentrations ($p<0.01$). (D) Zeta potential of Fc 400 also presented low negative values, with a significant decrease at 100 mg/ml ($p<0.001$). (E) Hydrodynamic radius of Fc 400 decreased significantly with increasing concentration ($p<0.05$), and its polydispersity (F) was found to increase ($p<0.001$). For Fc 1,000, significantly lower charge was found at 2.25 and 5 mg/ml (G) and the hydrodynamic radius (H) and polydispersity (I) did not change with concentration.
Figure 2.7: Collagen I deposition at various concentrations of Fc 70, 400 and 1,000 kDa. SDS-PAGE for collagen type I deposited by adult dermal fibroblasts at 3, 7 and 14 days of culture in presence of increasing concentrations of Fc 70 (A), 400 (B) and 1,000 kDa (C) and a non-crowded control (-MMC). Increased deposition was found at day 14 for Fc 70 at 37.5 mg/ml and for Fc 400 at 25 mg/ml. Fc 1,000 did not show any deposition of collagen I.
Figure 2.8: Densitometric analysis of collagen $\alpha$1(I) bands of SDS-PAGE gels representing collagen I deposition in the presence of various concentrations of Fc 70 kDa (A) and 400 kDa (B) and a non-crowded control (-MMC) at days 3, 7 and 14.
Figure 2.9: Immunocytochemistry images showing deposition of collagen type I (A) and collagen type III (B) at 3, 7 and 14 days using different concentrations of Fe 70 kDa.
Figure 2.10: Immunocytochemistry images showing deposition of collagen type I (A) and collagen type III (B) at 3, 7 and 14 days using different concentrations of Fc 400 kDa.
Figure 2.11: Immunocytochemistry images showing deposition of collagen type I (A) and collagen type III (B) at 3, 7 and 14 days using different concentrations of Fc 1,000 kDa.
Figure 2.12: Cellular morphology (A), viability (B) and metabolic activity (C) of adult dermal fibroblasts at days 3, 7 and 14 with varying concentrations of Fc 70 kDa. Cells maintained their spindle shaped morphology throughout the time points and metabolic activity was not significantly affected. Viability was also unaffected (green cells: live; dead cells: red).
Figure 2.13: Cellular morphology (A), viability (B) and metabolic activity (C) of adult dermal fibroblasts at days 3, 7 and 14 with varying concentrations of Fc 400 kDa. Cells maintained their spindle shaped morphology throughout the time points and metabolic activity was not significantly affected. Viability was also unaffected (green cells: live; dead cells: red).
Figure 2.14: Cellular morphology (A), viability (B) and metabolic activity (C) of adult dermal fibroblasts at days 3, 7 and 14 with varying concentrations of Fe 1,000 kDa. Cells maintained their spindle shaped morphology throughout the time points and metabolic activity was not significantly affected. Viability was also unaffected (green cells: live; dead cells: red).
2.3.3 Dextran sulphate (DxS)

The zeta potential of the various DxS molecules was negative throughout all concentrations and molecular weights (Figure 2.15A, Figure 2.15D and Figure 2.15G). The charge of DxS 10 was found to decrease significantly ($p<0.001$) with increasing its concentration between 10 and 500 $\mu$g/ml (Figure 2.15A). The charge of DxS 100 was found to decrease significantly ($p<0.05$) with increasing its concentration between 10 and 500 $\mu$g/ml (Figure 2.15D). The charge of DxS 500 was found to decrease significantly ($p<0.05$) at 500 and 1,000 $\mu$g/ml (Figure 2.15G). The hydrodynamic radius and polydispersity of several conditions were not attainable due to the limited sensitivity of the equipment used. For DxS 500, the concentration did not affect significantly ($p>0.05$) the hydrodynamic radius (Figure 2.15H), whilst the polydispersity decreased significantly ($p<0.05$) between 100 and 1,000 $\mu$g/ml (Figure 2.15I). Considering that several values were unattainable for the hydrodynamic radius of DxS 10 and 100, FVO values were only calculated for DxS 500. FVO increased linearly with increasing the concentration of DxS 500, peaking at 20% at 1,000 $\mu$g/ml (Figure 2.2E). SDS-PAGE (Figure 2.16A) revealed that by day 14, no collagen had been deposited when DxS 10 was used at various concentrations. SDS-PAGE (Figure 2.16B) and complementary densitometric analysis (Figure 2.17A) revealed no statistical difference ($p>0.05$) in collagen deposition between the various concentrations of DxS 100. SDS-PAGE (Figure 2.16C) and complementary densitometric analysis (Figure 2.17B) made apparent that 50 and 100 $\mu$g/ml DxS 500 significantly increased ($p<0.001$) collagen I deposition. Immunocytochemistry analysis confirmed the absence of collagen type I in the cell layer of groups treated with increasing concentrations of DxS 10 (Figure 2.18A). Collagen type III was detected in very low amounts at days 7 and 14 and DxS 10 did not lead to an increase in its presence (Figure 2.18B). DxS 100 led to increased collagen type I for concentrations between 10 and 100 $\mu$g/ml, as seen by immunocytochemistry (Figure 2.19A), whilst collagen type III was found in low amounts throughout all concentrations and time points (Figure 2.19B). Immunocytochemistry and SDS-PAGE findings for DxS 500 were concordant: deposition of collagen type I was increased between 10 and 250 $\mu$g/ml, particularly at day 14 (Figure 2.20A). Collagen type III was detected in higher amounts than observed with DxS 10 and 100 and was predominantly increased at
10 and 50 μg/ml (Figure 2.20B). Cell morphology (A), viability (B; \( p > 0.05 \)) and metabolic activity (C; \( p > 0.05 \)) were not affected by the various concentrations of different molecular weight DxS 10 (Figure 2.21), DxS 100 (Figure 2.22) and DxS 500 (Figure 2.23).
Figure 2.15: DLS measurement for different molecular weight DxS molecules (10, 100 and 500 kDa) at different concentrations. (A) Charge of DxS 10 presented a significant decrease with increasing concentrations between 10 and 500 µg/ml ($p<0.001$). Charge of 100
(D) showed an overall decrease with increasing concentrations \((p<0.05)\). (G) Zeta potential of DxS 500 decreased significantly at 500 and 1,000 \(\mu\text{g/ml}\) \((p<0.05)\). (B, E, H) Several measurements were not attainable due to the sensitivity of the equipment used. (H) Hydrodynamic radius of DxS 500 was not affected and its polydispersity index (I) decreased significantly between 100 and 1,000 \(\mu\text{g/ml}\) \((p<0.05)\).
Figure 2.16: Collagen I deposition at various concentrations of DxS 10, 100 and 500 kDa. SDS-PAGE for collagen type I deposited by adult dermal fibroblasts at 3, 7 and 14 days of culture in presence of increasing concentrations of DxS 10 (A), 100 (B) and 500 kDa (C) and a non-crowded control (-MMC). (C) 50 µg/ml of DxS 500 showed increased collagen I deposition at day 14.
Figure 2.17: Densitometric analysis of collagen α1(I) bands of SDS-PAGE gels representing collagen I deposition in the presence of various concentrations of 100 kDa (A) and 500 kDa (B) and a non-crowded control (-MMC) at days 3, 7 and 14
Figure 2.18: Immunocytochemistry images showing deposition of collagen type I (A) and collagen type III (B) at 3, 7 and 14 days using different concentrations of DxS 10 kDa.
Figure 2.19: Immunocytochemistry images showing deposition of collagen type I (A) and collagen type III (B) at 3, 7 and 14 days using different concentrations of DxsS 100 kDa.
Figure 2.20: Immunocytochemistry images showing deposition of collagen type I (A) and collagen type III (B) at 3, 7 and 14 days using different concentrations of DxS 500 kDa
Figure 2.21: Cellular morphology (A), viability (B) and metabolic activity (C) of adult dermal fibroblasts at days 3, 7 and 14 with varying concentrations of DxS 10 kDa. Cells maintained their spindle shaped morphology throughout the time points and metabolic activity was not significantly affected. Viability was also unaffected (green cells: live; dead cells: red).
Figure 2.22: Cellular morphology (A), viability (B) and metabolic activity (C) of adult dermal fibroblasts at days 3, 7 and 14 with varying concentrations of DxS 100 kDa. Cells maintained their spindle shaped morphology throughout the time points and metabolic activity was not significantly affected. Viability was also unaffected (green cells: live; dead cells: red).
Figure 2.23: Cellular morphology (A), viability (B) and metabolic activity (C) of adult dermal fibroblasts at days 3, 7 and 14 with varying concentrations of DxS 500 kDa. Cells maintained their spindle shaped morphology throughout the time points and metabolic activity was not significantly affected. Viability was also unaffected (green cells: live; dead cells: red).
2.3.4 Mixed crowding using Ficoll™ (Fc) cocktails
Cocktails of different molecular weight Fc molecules were prepared by varying the concentration of one molecule and maintaining the other two constant. Optimal concentrations of Fc 70 (37.5 mg/ml) and 400 (25 mg/ml) from the previous section were used here. Since Fc 1,000 did not induce deposition of collagen type I, the concentration to be kept constant (2.25 mg/ml) was the one determined as theoretically optimal by a linear regression. Increasing the concentration of Fc 1,000 in a cocktail with Fc 70 (37.5 mg/ml) and Fc 400 (25 mg/ml) did not affect ($p>0.05$) the charge (Figure 2.24A). Increasing the concentration of Fc 400 in a cocktail with Fc 70 (37.5 mg/ml) and Fc 1,000 (2.25 mg/ml) did not affect ($p>0.05$) the charge (Figure 2.24D). The zeta potential increased significantly ($p<0.05$) at 50 mg/ml of Fc 70 in a mixture with constant Fc 400 (25 mg/ml) and Fc 1,000 (2.25 mg/ml) (Figure 2.24G). Hydrodynamic radius analysis revealed that all three-molecule cocktails were comprised of three-distinct particle sizes: 5 to 10 nm, 20 to 60 nm and >1,000 nm (Figure 2.24B, Figure 2.24E and Figure 2.24H). For cocktails of varying concentrations of Fc 1,000 and constant Fc 70 (37.5 mg/ml) and Fc 400 (25 mg/ml), polydispersity was increased significantly ($p<0.05$) at 10 mg/ml of Fc 1,000 (Figure 2.24C). Variable concentrations of Fc 400 in a constant Fc 70 (37.5 mg/ml) and Fc 1,000 (2.25 mg/ml) solution, did not significantly ($p>0.05$) affect polydispersity (Figure 2.24F). Polydispersity increased significantly ($p<0.01$) with increasing concentrations of Fc 70 in a cocktail with constant Fc 400 (25 mg/ml) and Fc 1,000 (2.25 mg/ml) (Figure 2.24I). SDS-PAGE (Figure 2.25A) and complementary densitometric analysis (Figure 2.26A) revealed that collagen I deposition was not significantly ($p>0.05$) enhanced when variable concentrations of Fc 1,000 were added to a mixture of constant Fc 70 (37.5 mg/ml) and Fc 400 kDa (25 mg/ml). SDS-PAGE (Figure 2.25B) and complementary densitometric analysis (Figure 2.26B) revealed that collagen I deposition was not significantly ($p>0.05$) enhanced when variable concentrations of Fc 400 were added to a mixture of constant Fc 70 (37.5 mg/ml) and Fc 1,000 kDa (2.25 mg/ml). SDS-PAGE (Figure 2.25C) and complementary densitometric analysis (Figure 2.26C) revealed that collagen I deposition was significantly ($p<0.05$) increased when 10 mg/ml Fc 70 were added to a mixture of constant Fc 400 (25 mg/ml) and Fc 1,000 kDa (2.25 mg/ml), though comparable to Fc 70 / 400. Contradictory to SDS-PAGE data, immunocytochemistry revealed absence of collagen type I at all time points.
when different concentrations of Fc 1,000 were combined with constant Fc 400 (25 mg/ml) and Fc 70 (37.5 mg/ml) (Figure 2.27A). In contrast, collagen type III was increased in all groups when compared to -MMC counterparts (Figure 2.27B). The same trend was observed with cocktails of varying concentrations of Fc 400 and constant Fc 1,000 (2.25 mg/ml) and Fc 70 (37.5 mg/ml) (Figure 2.28) and cocktails of varying concentrations of Fc 70 and constant Fc 1,000 (2.25 mg/ml) and Fc 400 (25 mg/ml, Figure 2.29).

Cell morphology (A), viability (B; \( p > 0.05 \)) and metabolic activity (C; \( p > 0.05 \)) were not affected by varying the: Fc 1,000 concentration in a constant Fc 70 (37.5 mg/ml) and Fc 400 (25 mg/ml) solution (Figure 2.30); Fc 400 concentration in a constant Fc 70 (37.5 mg/ml) and Fc 1,000 (2.25 mg/ml) solution (Figure 2.31); and Fc 70 concentration in a constant Fc 400 (25 mg/ml) and Fc 1,000 (2.25 mg/ml) solution (Figure 2.32).
Figure 2.24: DLS measurements of cocktails of different molecular weight Fc molecules (70, 400 and 1,000 kDa). (A) Zeta potential was not significantly affected by the presence of increasing concentrations of Fc 1,000 kDa with constant Fc 70 kDa and 400 kDa. (B)
Three particle sizes of 5 – 10 nm, 40 – 60 nm and ~2000 nm were found. (C) PDI was increased significantly at 10 mg/ml of Fc 1,000 ($p<0.05$). (D) Zeta potential of varying concentration of Fc 400 with constant Fc 70 and 1,000 did not change significantly. (E) Three particle sizes of 5 – 10 nm, 40 – 60 nm and >1,000 nm were found. (F) PDI was increased at 50 mg/ml of Fc 400 ($p<0.05$). (G) Increasing concentrations of Fc 70 in constant Fc 400 and 1,000, caused a significant increase in zeta potential up to 50 mg/ml after which it decreases. (H) Three particle sizes of 5 – 10 nm, 20 – 40 nm and >1,000 nm were found. (I) PDI increased significantly with increasing concentration of Fc 70 in solution ($p<0.01$).
Figure 2.25: Collagen I deposition under mixed Fc crowding. SDS-PAGE for collagen type I deposited by adult dermal fibroblasts at 3, 7 and 14 days of culture in presence of cocktails of different molecular weight Fc molecules (70, 400 and 1,000 kDa) and a non-crowded control (-MMC). (A, B) Deposition of collagen type I was similar between -MMC and remaining groups. (C) 10 mg/ml of Fc 70 with constant Fc 400 and 1,000 also exhibited increased collagen I deposition at day 14 over -MMC ($p<0.05$), but not significantly different from Fc 70 / 400.
**Figure 2.26:** Densitometric analysis of collagen $\alpha_1$(I) bands of SDS-PAGE gels representing collagen I deposition in the presence of various concentrations of Fc 1,000 kDa (A), 400 kDa (B) and 70 kDa (C) in cocktails and a non-crowded control (-MMC) at days 3, 7 and 14.
Figure 2.27: Immunocytochemistry images showing deposition of collagen type I (A) and collagen type III (B) at 3, 7 and 14 days using different concentrations of Fc 1,000 kDa in cocktails with constant Fc 400 kDa and 70 kDa.
Figure 2.28: Immunocytochemistry images showing deposition of collagen type I (A) and collagen type III (B) at 3, 7 and 14 days using different concentrations of Fc 400 kDa in cocktails with constant Fc 70 kDa and 1,000 kDa.
Figure 2.29: Immunocytochemistry images showing deposition of collagen type I (A) and collagen type III (B) at 3, 7 and 14 days using different concentrations of Fc 70 kDa in cocktails with constant Fc 400 kDa and 1,000 kDa.
Figure 2.30: Cellular morphology (A), viability (B) and metabolic activity (C) of adult dermal fibroblasts at days 3, 7 and 14 with varying concentrations of Fc 1,000 kDa in cocktails with constant Fc 70 kDa and 400 kDa. Cells maintained their spindle shaped morphology throughout the time points and metabolic activity was not significantly affected. Viability was also unaffected (green cells: live; dead cells: red).
Figure 2.31: Cellular morphology (A), viability (B) and metabolic activity (C) of adult dermal fibroblasts at days 3, 7 and 14 with varying concentrations of Fc 400 kDa in cocktails with constant Fc 70 kDa and 1,000 kDa. Cells maintained their spindle shaped morphology throughout the time points and metabolic activity was not significantly affected. Viability was also unaffected (green cells: live; dead cells: red).
Figure 2.32: Cellular morphology (A), viability (B) and metabolic activity (C) of adult dermal fibroblasts at days 3, 7 and 14 with varying concentrations of Fc 70 kDa in cocktails with constant Fc 400 kDa and 1,000 kDa. Cells maintained their spindle shaped morphology throughout the time points and metabolic activity was not significantly affected. Viability was also unaffected (green cells: live; dead cells: red).
2.3.5 Mixed crowding using dextran sulphate (DxS) cocktails

Cocktails of different molecular weight DxS were also used to achieve mixed crowding. Similarly to Fc, cocktails of different molecular weight DxS molecules were prepared by varying the concentration of one molecule and maintaining the other two constant. Concentrations to maintain constant were chosen based on the range of optimal individual performance in terms of collagen type I deposition (DxS 500, 100 µg/ml) or, when significant differences were not found, based on range of concentrations used in previous literature for dextran sulphate molecules (DxS 10 and DxS 100, 100 µg/ml) [49, 52-54]. The zeta potential was not statistically altered ($p>0.05$) when variable concentrations of DxS 500 were added to a constant DxS 10 (100 µg/ml) and DxS 100 (100 µg/ml) solution (Figure 2.33A). The zeta potential was also not statistically altered ($p>0.05$) when variable concentrations of DxS 100 were added to a constant DxS 10 (100 µg/ml) and DxS 500 (100 µg/ml) solution (Figure 2.33D). The zeta potential was statistically increased ($p<0.05$) only at 0 µg/ml DxS 10 and at constant DxS 100 (100 µg/ml) and DxS 500 (100 µg/ml) solution (Figure 2.33G). Hydrodynamic radius measurements of the various DxS cocktails revealed highly variable particle size (Figure 2.33B, Figure 2.33E and Figure 2.33H). Some values were not detected, as they were below (< 0.3 nm) or above (> 5 µm) the detection limit. Polydispersity was significantly decreased ($p<0.001$) when 0 and 500 µg/ml DxS 500 were used in a solution of constant DxS 10 (100 µg/ml) and DxS 100 (100 µg/ml) (Figure 2.33C). Polydispersity was significantly increased ($p<0.05$) when 1,000 µg/ml DxS 10 were used in a solution of constant DxS 10 (100 µg/ml) and DxS 500 (100 µg/ml) (Figure 2.33F). Polydispersity was significantly decreased ($p<0.05$) when 10 and 500 µg/ml DxS 10 were used in a solution of constant DxS 100 (100 µg/ml) and DxS 500 (100 µg/ml) (Figure 2.33I). SDS-PAGE (Figure 2.34A) and complementary densitometric analysis (Figure 2.35A) revealed that collagen type I deposition was decreased ($p<0.05$) when 500 µg/ml DxS 500 were used in a solution of constant DxS 10 (100 µg/ml) and DxS 100 (100 µg/ml) at all time points; remaining groups did not exhibit significant ($p>0.05$) fold-change over the –MMC counterparts. SDS-PAGE (Figure 2.34B) and complementary densitometric analysis (Figure 2.35B) revealed that variable concentrations of DxS 100 with constant DxS 10 (100 µg/ml) and DxS 500 (100 µg/ml) did not induce enhanced ($p>0.05$) deposition when compared to the –MMC counterparts. SDS-
PAGE (Figure 2.34C) and complementary densitometric analysis (Figure 2.35C) revealed that the highest ($p<0.05$) collagen type I deposition was observed when 10 $\mu$g/ml DxS 10 were used in a solution of constant DxS 100 (100 $\mu$g/ml) and DxS 500 (100 $\mu$g/ml) at day 3. Immunocytochemical analysis revealed presence of collagen type I at all time points, predominantly when the concentration of DxS 500 was lower than 100 $\mu$g/ml with constant DxS 100 (100 $\mu$g/ml) and DxS 10 (100 $\mu$g/ml) (Figure 2.36A). Collagen type III was also detected throughout all conditions, though it was decreased at day 14 (Figure 2.36B). When different concentrations of DxS 100 were used with constant DxS 500 (100 $\mu$g/ml) and DxS 10 (100 $\mu$g/ml), collagen type I was detected only at day 3 (Figure 2.37A), whilst low amounts of collagen type III were present at all time points (Figure 2.37B). Similarly, at concentrations lower than 100 $\mu$g/ml of DxS 10 and constant DxS 500 (100 $\mu$g/ml) and DxS 100 (100 $\mu$g/ml), low quantities of collagen type I (Figure 2.38A) and III (Figure 2.38B) were detected. Cell morphology (A), viability (B; $p>0.05$) and metabolic activity (C; $p>0.05$) were not affected by varying the: DxS 500 concentration in a constant DxS 10 (100 $\mu$g/ml) and DxS 100 (100 $\mu$g/ml) solution (Figure 2.39); DxS 100 concentration in a constant DxS 10 (100 $\mu$g/ml) and DxS 500 (100 $\mu$g/ml) solution (Figure 2.40); and DxS 10 concentration in a constant DxS 100 (100 $\mu$g/ml) and DxS 500 (100 $\mu$g/ml) solution (Figure 2.41).
Figure 2.33: DLs measurements for cocktails of different molecular weight DxS molecules (10, 100 and 500 kDa). (A) Charge did not change significantly when increasing the concentration of DxS 500 with constant DxS 10 and 100. (B, E, H) Hydrodynamic radius was
found to be extremely variable and three different particle sizes were observed. (C) PDI was significantly decreased at 0 and 500 µg/ml of DxS 500 ($p<0.001$). (D) Charge of varying concentrations of DxS 100 with constant DxS 10 and 500 did not change significantly. (F) PDI at 1,000 µg/ml was significantly increased ($p<0.05$). (G) Zeta potential was increased in the absence of DxS 10 (0 µg/ml) with constant DxS 100 and 500 ($p<0.05$). (I) PDI was significantly lower at 10 and 500 µg/ml of DxS 10 ($p<0.05$).
Figure 2.34: Collagen I deposition under mixed Dxs crowding. SDS-PAGE for collagen type I deposited by adult dermal fibroblasts at 3, 7 and 14 days of culture in presence of cocktails of different molecular weight Dxs molecules (10, 100 and 500 kDa) and a non-crowded control (-MMC). (A) Increased deposition of collagen I was found at 50 µg/ml of Dxs 500 with constant Dxs 10 and 10; (B) at 50 µg/ml of Dxs 100 with constant Dxs 10 and 500; and (C) at 10 µg/ml of Dxs 10 with constant Dxs 100 and 500, all at day 14.
Figure 2.35: Densitometric analysis of collagen α1(I) bands of SDS-PAGE gels representing collagen I deposition in the presence of various concentrations of DxS 500 kDa (A), 100 kDa (B) and 10 kDa (C) in cocktails and a non-crowded control (-MMC) at days 3, 7 and 14.
Figure 2.36: Immunocytochemistry images showing deposition of collagen type I (A) and collagen type III (B) at 3, 7 and 14 days using different concentrations of DxS 500 kDa in cocktails with constant DxS 10 kDa and 100 kDa.
Figure 2.37: Immunocytochemistry images showing deposition of collagen type I (A) and collagen type III (B) at 3, 7 and 14 days using different concentrations of Dxs 100 kDa in cocktails with constant Dxs 10 kDa and 500 kDa.
Figure 2.38: Immunocytochemistry images showing deposition of collagen type I (A) and collagen type III (B) at 3, 7 and 14 days using different concentrations of DxS 10 kDa in cocktails with constant DxS 100 kDa and 500 kDa.
Figure 2.39: Cellular morphology (A), viability (B) and metabolic activity (C) of adult dermal fibroblasts at days 3, 7 and 14 with varying concentrations of Dxs 500 kDa in cocktails with constant Dxs 10 kDa and 100 kDa. Cells maintained their spindle shaped morphology throughout the time points and metabolic activity was not significantly affected. Viability was also unaffected (green cells: live; dead cells: red).
Figure 2.40: Cellular morphology (A), viability (B) and metabolic activity (C) of adult dermal fibroblasts at days 3, 7 and 14 with varying concentrations of DxS 100 kDa in cocktails with constant DxS 10 kDa and 500 kDa. Cells maintained their spindle shaped morphology throughout the time points and metabolic activity was not significantly affected. Viability was also unaffected (green cells: live; dead cells: red).
Figure 2.41: Cellular morphology (A), viability (B) and metabolic activity (C) of adult dermal fibroblasts at days 3, 7 and 14 with varying concentrations of DxS 10 kDa in cocktails with constant DxS 100 kDa and 500 kDa. Cells maintained their spindle shaped morphology throughout the time points and metabolic activity was not significantly affected. Viability was also unaffected (green cells: live; dead cells: red).
2.3.6 Direct comparison of optimal crowding conditions

SDS-PAGE for the optimal conditions of each crowder (CR: 100 μg/ml; Fc: 70 at 37.5 mg/ml and 400 at 25 mg/ml; DxS: 10 at 10 μg/ml, 100 at 100 μg/ml, 500 at 100 μg/ml) at day 3 demonstrated that CR induced the highest ($p<0.001$) collagen type I deposition (Figure 2.42).
Figure 2.42: Optimal conditions for each group of crowders at day 3. CR (100 μg/ml), Fe 70 and 400 cocktail (37.5 and 25 mg/ml, respectively) and DxS 10, 100 and 500 (10, 100 and 100 μg/ml respectively). CR presented the highest collagen I deposition.
2.4 Discussion

Advancements in the tissue engineering field have facilitated the development of tissue-like structures derived from the cells’ innate ability to build complex supramolecular assemblies [55, 56]. Although significant progress has been achieved using various microenvironmental cues, none of these approaches accelerates ECM deposition, which results in prolonged culture periods, leading to phenotypic drift and loss of cells’ therapeutic potential, thereby hindering the development of modular cell-based implantable devices. Herein, we ventured to assess the influence of crowder and crowder’s concentration, molecular weight, charge, hydrodynamic radius and polydispersity on ECM deposition of human primary adult dermal fibroblasts.

DLS analysis of different CR concentrations, clearly demonstrated a concentration-dependent decrease in surface charge and increase in hydrodynamic radius and polydispersity, confirming previous reports of negative charge and high polydispersity [57, 58]. Variations in zeta potential with particle concentration have been previously described in the literature, although it is not clear if such results are reliable or an artefact [59, 60]. A previous study suggests there is a range of concentrations, typically in more dilute solutions, where the zeta potential is dependent on concentration and that this should be avoided for accurate measurements [59]. Further, due to the nature of the DLS system, measurements can be affected at lower (a weaker signal might lead to light scattering from other sources to be detected) and higher concentrations (as concentration and PDI increase, more signal might derive from extraneous particles).

SDS-PAGE and ICC analysis also showed a concentration-dependent increase in collagen deposition. In a dilute culture environment, the newly synthesised water soluble procollagen is dissolved before the $N$- and $C$-propeptides are cleaved by their respective proteinases. Adding inert macromolecules to the culture medium restricts molecule diffusion, facilitating cleavage of the propeptide extensions by the $N$- and $C$- proteinases, which accelerates ECM deposition [29, 43, 49]. Here, increasing the concentration of CR created a more confined environment, as demonstrated by the exponentially increasing FVO, that optimally enhanced ECM deposition at 100 $\mu$g/ml. This derives from electrostatic interactions that affect the molecules’ hydration shell and lead to increased repulsion effects, all contributing towards changes in the hydrodynamic radius, charge and polydispersity. Higher
concentrations created an over-crowding effect that hinders molecule diffusion in solution, therefore slowing down enzymatic reactions, as seen from the exponential increase in FVO and plateauing/decrease in collagen I deposition at 500 µg/ml of CR. Accelerated ECM deposition in the presence of CR confirms reports of previous results obtained with WS-1 fibroblasts [44], bone marrow derived mesenchymal stem cells [45] and corneal fibroblasts [53] at a similar concentration range.

The different molecular weight Fc displayed overall a neutral or nominal negative charge; unchanged or, in the case of Fc 400, decreased the hydrodynamic radius with concentration; and increased polydispersity for Fc 70 and 400. The radius of Fc 70 was within the range previously obtained in the literature for similar concentrations [61, 62], whilst the radius of Fc 400 contradicts former results, as it varies widely with concentration [28, 49, 61]. However, it is important to consider that most studies only measure the radius at one concentration. SDS-PAGE exhibited higher collagen deposition only at day 14 at 37.5 and 25 mg/ml for Fc 70 and 400 respectively. However, this represented a very small improvement over the -MMC counterparts. These results are concordant with published data using these molecules separately at 50 mg/ml on WI-38 fibroblasts with no significant enhancement of collagen deposition [49]. Fc 1,000 did not induce deposition of collagen type I, and based on the theory of the excluding volume effect, this relates with its large particle size and low polydispersity, which, although conducive to a high theoretical FVO, experimentally translates into an over-crowding effect. The decrease in the hydrodynamic radius of Fc 400 has been described in previous literature as a consequence of intermolecular interactions that occur at high concentrations and prompt intra-chain folding events [61]. ICC analysis revealed inconsistent data when compared with SDS-PAGE, showing absence of collagen I and presence of collagen type III at all time points. However, SDS-PAGE was performed under non-reducing conditions, meaning that the α1(III) likely co-migrates with the γ(I) chain [63]. To be able to separate these bands and confirm the presence of type III collagen, delayed and reduced electrophoresis can be used to dissociate the interchain disulphide bonds present in collagen type III using 2-mercaptoethanol, cyanogen bromide or urea [63-65]. Types I and III collagen are commonly found together forming heterotypic fibrils, with type III being typically localised at the periphery of the fibrils, suggesting it has an important role in
regulation of fibril growth, diameter and interactions with other ECM molecules [66]. Moreover, the type I procollagen C-proteinase involved in extracellular processing of procollagen I also cleaves the C-terminal of the α1(III) propeptide at a similar rate [67]. In healthy dermal cells, it has also been demonstrated that, after 3 days of supplementation with ascorbic acid, procollagen I is found mostly inside the cell, whilst procollagen III is found in higher amounts in the extracellular space, suggesting the post-translational processing events that form insoluble collagen III occur earlier [68]. Overall, this correlates with ICC findings of earlier deposition of collagen type III, independently of MMC and altered band density of the gels. Additionally, the detection limits of the two techniques differ substantially: the silver staining procedure for SDS-PAGE, according with the manufacturer, can detect protein at the sub-nano-gram level, whilst detection by ICC, due to variability associated with the use of antibodies and the recognised immunogen sequence and conformation (used antibody detected the full length native protein), will more than likely be less sensitive. The discrepancy between the two techniques raises questions regarding the effective binding of the primary antibody. The primary antibody used was tested in-house for ICC using other cells known to deposit collagen type I (WS-1 and WI-38 fibroblasts). Nonetheless, tissue, such as skin or tendon, could have also been used to further validate the antibody. Another issue could be epitope availability for binding due to masking by other molecules or cross-linking present in the cell layer or due to alterations in conformation, electrostatic charges or cross-linking during the fixation step with paraformaldehyde. These issues are more common when working with tissue and are typically overcome by adding an antigen retrieval step to the staining protocol [69, 70], therefore, further optimisation would be required with regards to the use of this specific primary antibody for collagen type I.

Continuing with the DLS analysis of different molecular weight DxS, when measurements were possible, an overall negative charge was observed and, for DxS 500, polydispersity was found to decrease with increasing concentrations. The hydrodynamic radius of DxS 500 was contradictory with previous reports for similar concentrations [61]. Hydrodynamic radius measurements of DxS 10 were also previously attempted with this technique and, likewise, no data was attainable [49]. Since several measurements were not attainable due to equipment limitations, other techniques, such as nanoparticle tracking analysis must be considered to
investigate the biophysical properties of DxS in physiological solutions. SDS-PAGE demonstrated that collagen I deposition was only found when DxS 100 and 500 were used. Previous work with DxS 10 at 100 µg/ml, corroborates these findings, as collagen deposition was not enhanced in WI-38 fibroblast culture [49]. DxS 500 was shown to work optimally at 50 and 100 µg/ml, again confirming earlier work with WI-38 fibroblasts [71] and dermal fibroblasts [43]. ICC analysis exhibited concordant data with minimal deposition of collagen type I and some collagen type III present.

Since our data indicated that polydispersity and the consequent increase in FVO are closely related with higher collagen deposition, mixed crowding was applied to create artificial polydispersity in the culture environment. Mixed crowding has also been used to analyse protein stabilisation [35] and refolding [72], stabilisation of complementary nucleotide binding during PCR [73] and enhancing ECM deposition [52, 71]. Mixed crowding has been utilised either as a combination of the same molecules in different molecular weights (e.g. Fc 70 and 400) [47, 71, 74] or as a mixture of different molecules (e.g. Fc and DxS [52], dextran and bovine serum albumin [72]). Taking the individual optimal concentrations of Fc 70 (37.5 mg/ml) and Fc 400 (25 mg/ml), cocktails were prepared by adding a third molecule, Fc 1,000. All Fc cocktails were found to have very a small negative surface charge, comparable to the same molecules individually. Hydrodynamic radius measurements revealed the presence of particle sizes higher than the ones corresponding to the molecules alone, indicating particle aggregation in the solution, which led to increased polydispersity. Nonetheless, in terms of enhancement of ECM deposition, all cocktails presented similar results to the -MMC control. Further, there was no obvious advantage in adding the third higher molecular weight component (Fc 1,000) to the already established Fc 70 and 400 cocktail. Although Fc 1,000 had not been used before, Fc 70 and 400 have been combined with DxS 10 (100 µg/ml) to create ECM-rich decellularised substrates produced by human mesenchymal stem cells and enhanced ECM deposition was described [52]; it is important to note that, in that case, a smaller, negatively charged molecule was chosen to improve the performance of the Fc cocktail.

Dextran sulphate cocktails composed of three different molecular weight molecules were also prepared. Similarly to the individual molecules, a negative charge was found for all cocktails. Hydrodynamic radius measurements displayed highly
variable data, again indicative of particle aggregation [75, 76], which consequently increased the polydispersity of the solutions over the individual molecules. SDS-PAGE made clear that the highest fold-change over the -MMC counterparts was observed when varying concentrations of DxS 10 were used with constant DxS 100 (100 µg/ml) and 500 (100 µg/ml), as collagen I can be observed from day 3 onwards, particularly at 10 and 50 µg/ml. For all cocktails, ICC revealed similar amounts of collagen types I and III.

Overall, CR significantly accelerated ECM deposition, as its negative charge and natural polydispersity allowed for a more efficient volume exclusion. The neutral charge of Fc and the low polydispersity of DxS did not match the enhanced ECM deposition of CR. Although mixed crowding enabled higher polydispersity in Fc and DxS cocktails, the performance of CR was still unmatched, indicating that natural polydispersity is more efficient in creating crowded and more physiological environments that accelerate the rate of biological reactions. Natural polymers, after all, are generally more polydisperse than synthetic materials since in place manufacturing procedures ensure consistent chain lengths, high reproducible and low variability [77].
2.5 Conclusion

We assessed the influence of hydrodynamic radius, charge, polydispersity and concentration of different crowders (CR; Fc 70, 400, 1,000 kDa; and Dxs 10, 100, 500 kDa) on ECM deposition. Crowder concentration was found to affect the hydrodynamic radius, charge and polydispersity. To improve performance of the different Fc and Dxs, mixed crowding was utilised. All Fc and Dxs cocktails presented increased polydispersity over the same molecules individually. CR enhanced collagen I and III deposition most efficiently at 100 µg/ml and was unmatched by any of the Fc or Dxs molecules alone or in cocktail, due to its inherent negative charge and polydispersity. Our results highlight the importance of the crowder’s biophysical properties and contribute to a better understanding of how MMC affects biological reactions in vitro.
2.6 References


Chapter 3 – Macromolecular crowding and low oxygen tension for human mesenchymal stem cell phenotype maintenance and enhanced matrix deposition

Sections of this chapter have been published in:
3.1 Introduction
Numerous *in vitro* microenvironment modulators are at the forefront of scientific and technological research and innovation to either direct stem cells towards a specific lineage or to maintain stem cells’ and permanently differentiated cells’ phenotype during *ex vivo* expansion [1-3]. In particular, the ability to maintain stem cell function during *ex vivo* growth is fundamental for the development of reparative therapies, as their bioactive, trophic, immunomodulatory, angiogenic and anti-apoptotic secretome determines their therapeutic efficacy [4-6].

Given the complexity of the *in vivo* milieu, recent data advocate that multifactorial cell expansion approaches are likely to lead in clinically relevant cell therapies [7-10]. Among the various methods of microenvironmental induced signalling, physiological low oxygen tension has been shown to be of the utmost importance in maintaining stem cell phenotype, controlling their differentiation and fate and increasing their motility and therapeutic potential [11-14]. Further, through the activation of hypoxia inducible factor - 1α (HIF-1α), cell metabolism is regulated [15], cell cycle quiescence is maintained [16], angiogenesis is promoted [17] and ECM synthesis is enhanced [18, 19], which in turn has been shown to be crucial in regulating stem cell fate [20, 21]. However, in the physiologically irrelevant dilute culture media, this *de novo* synthesised ECM is dispersed and discarded during media changes. It has been recently demonstrated that the addition of inert and polydispersed macromolecules in culture media not only accelerates by up to 80-fold ECM deposition, but also maintains permanently differentiated cell phenotype, even at low density and low serum cultures [22-24]. This was attributed to macromolecular crowding (MMC) / excluding volume effect, a biophysical phenomenon that governs the physiological environment of multicellular organisms and intensifies biological processes and thermodynamic rates by several orders of magnitude [25, 26]. In a sense, MMC, by imitating the cell’s dense and confined native tissue context, accelerates biological processes, such as the enzymatic conversion of procollagen to collagen, which is onerous in the customarily used dilute culture conditions. To-date, although MMC has been shown to enhance and to organise ECM deposition in naïve stem cell culture [27-29] and to enhance adipogenesis in adipose-induced stem cells [30], its influence on naïve stem cell phenotype has yet to be demonstrated and no study has assessed the simultaneous effect of MMC and oxygen tension in the development of tissue-like
supramolecular assemblies. Here, we provide evidence that multifactorial approaches based on MMC and low oxygen tension significantly enhance ECM deposition and maintain the multipotent phenotype of human bone marrow mesenchymal stem cells (BMSCs) during \textit{in vitro} expansion.
3.2 Materials and Methods

3.2.1 Cell culture, macromolecular crowding and hypoxia

Human bone marrow derived stem cells were isolated from fresh bone marrow (Lonza) and culture in alpha – Minimum Essential Medium with GlutaMax™ (Gibco Life Technologies), supplemented with 10 % Hyclone™ foetal bovine serum (Thermo Scientific) and 1 % penicillin-streptomycin at 37 °C in a humidified atmosphere at 5 % CO₂. Cells were used between passages 2 and 4. For the various experiments, cells were cultured at 25,000 cells / cm² and allowed to attach for 24 hours, after which medium was changed to medium with 100 µM of L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate and macromolecular crowders (carrageenan at 1, 5, 10, 50, 100 and 500 µg/ml or a Ficoll™ cocktail containing 37.5 mg/ml of Ficoll™ 70 and 25 mg/ml of Ficoll™ 400. For low oxygen experiments, cells were maintained in an Oxygen Tissue Culture Glove Box (Coy Lab Products) at 2 % O₂ and 37 °C in a humidified atmosphere. Medium supplemented with crowders was changed every 3 days.

3.2.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

On the various time points, culture media was aspirated and cell layers were briefly washed with HBSS. Cell layers were then digested with pepsin from porcine gastric mucosa (Sigma, Ireland) at 0.1 mg / ml in 0.5 M acetic acid at 37 °C for 2 hours under agitation. Cell layers were then scraped and neutralised with 1 M sodium hydroxide.

Cell layer samples were analysed by SDS-PAGE under non-reducing conditions with a Mini-Protean 3 (Bio-Rad Laboratories, UK). Bovine collagen type I (100 µg / ml) was used as a standard for all gels. Staining of the protein bands was performed with the SilverQuest® kit following manufacturer’s instructions.

3.2.3 Immunocytochemistry

At each time point, cells were briefly washed with HBSS and fixed with 2 % paraformaldehyde (PFA) for 15 minutes at room temperature. Cell were washed again and non-specific site were blocked with 3 % bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 30 minutes. Cells were then incubated for 90 minutes at room temperature with primary antibody (collagen type I: ab90395;
collagen type III: ab7778 from Abcam, UK and fibronectin: F7387; laminin: L9393 from Sigma, Ireland), after which were washed 3 times with PBS, followed by 30 minutes of incubation at room temperature with secondary antibody. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescent images were captured with an inverted fluorescence microscope (Olympus IX-81, Olympus Corporation, Japan) and images were further processed with ImageJ software (NIIH, USA).

3.2.4 Total protein extraction and quantification
At each time point, cells were briefly washed with HBSS. RIPA buffer with proteinase inhibitor cocktail was added to the cell layer and left to incubate at 4 °C for 30 minutes, after which cell layer was collected by scratching, centrifuged and frozen at -80 °C. Total protein quantification was performed using the Pierce™ BCA Protein Assay Kit following manufacturer’s instructions. Protein concentration was determined using a BSA standard curve.

3.2.5 Sircol™ assay
Total collagen quantification was performed using the Sircol™ soluble collagen assay (Biocolor, UK) following manufacturer’s instructions. Briefly, reagent blanks and collagen standards were prepared, samples were digested with pepsin from porcine gastric mucosa (Sigma, Ireland) at 0.1 mg / ml in 0.5 M acetic acid at 37 °C for 2 hours under agitation. Sircol dye reagent was added to the samples and left on a shaker for 30 minutes. Samples were then transferred to tubes and centrifuged at 12,000 rpm for 10 minutes and supernatant was discarded. The acid-salt wash reagent was added to the pellets and samples were centrifuged again at 12,000 rpm for 10 minutes after which the supernatant was discarded. The alkali reagent was added to the samples to dissolve the sample-bound dye into solution and absorbance was measured at 555 nm using a Varioskan Flash Spectral scanning multimode reader (Thermo Fisher Scientific, UK).
3.2.6 Trilineage differentiation

Cells were cultured either with crowded medium containing 100 µg/ml carrageenan or with CTR medium, at either 20 % or 2 % O₂. After two weeks, cells cultured under the four conditions (CTR 20 % O₂, CTR 2 % O₂, C100 20 % O₂ and C100 20 % O₂) were harvested and fed with differentiating media at 20 % O₂. Differentiation ability was determined by histological and biochemical analysis. For each assay, negative controls were fed with complete medium. Adipogenic, osteogenic and chondrogenic differentiation were induced (BulletKit™, Lonza) per manufacturer’s protocol. Adipogenic differentiation was evaluated by Oil Red O staining and uptake quantification. Osteogenic differentiation was assessed by Alizarin Red S staining and uptake quantification. Chondrogenic differentiation was analysed by Safranin O / Fast Green staining and GAG quantification using 1,9-dimethylmethylen blue (DMMB) method. GAG content of pellets was normalised to DNA amount and expressed as µg per µg DNA.

3.2.7 Cell viability

Cell viability was evaluated using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega), following manufacturer’s instructions. Conditioned media were collected and incubated with the Substrate Mix at room temperature. After 30 minutes, absorbance was measured at 490 nm in a plate reader (Varioskan Flash, Thermo Scientific). Cell viability was expressed as percent difference between MMC-treated cells and untreated cells at day 2.

3.2.8 Statistical analysis

Data is expressed as mean ± standard deviation. Statistical analysis was performed using MINITAB version 16 (Minitab Inc., USA). One-way analysis of variance (ANOVA) was used for multiple comparisons and 2 sample t-test was used for pairwise comparisons after confirming the samples followed a normal distribution (Kolmogorov-Smirnov) and had equal variances (Bartlett’s and Levene’s test for homogeneity of variances). When one of these assumptions was violated, non-parametric tests were used for multiple comparisons (Kruskall-Wallis test) and pairwise comparisons (Mann-Whitney test). Statistical significance was accepted at p<0.05.
3.3 Results
To assess the influence of MMC on ECM deposition, different concentrations of carrageenann (CR) were assessed and compared with a Ficoll™ cocktail (FC) and a non-MMC control (CTR). Collagen type I deposition was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Figure 3.1A) and corresponding densitometric analysis (Figure 3.1B) revealed that CR concentrations in the range of 50 to 500 μg/ml induced the highest (p<0.001) collagen type I deposition at all time points assessed (2, 4, 7 and 14 days). Immunocytochemistry demonstrated enhanced collagen type I (Figure 3.2), collagen type III (Figure 3.3), fibronectin (Figure 3.4) and laminin (Figure 3.5) deposition in the presence of 100 and 500 μg/ml CR after 4, 7 and 14 days in culture. Cell viability (Figure 3.6) was not affected, independently of the treatment and time in culture. To assess the simultaneous effect of oxygen tension and MMC on ECM deposition, BMSCs were cultured at 20 % and 2 % oxygen tension in the absence (CTR) and presence of 100 μg/ml CR. SDS-PAGE of the cell layers (Figure 3.7) revealed a significantly higher (p<0.001) ECM deposition between the CR and CTR groups at both oxygen tensions (20 % and 2 %) and time points (7 and 14 days), but no significant difference (p>0.05) was observed in collagen type I deposition between the MMC and non-MMC groups at 20 % and 2 % oxygen tension and both time points (7 and 14 days). Immunocytochemistry further corroborated these observations, as a higher collagen type I (Figure 3.8A), collagen type III (Figure 3.8B), fibronectin (Figure 3.9A) and laminin (Figure 3.9B) deposition was observed between the CR and CTR groups at both oxygen tensions (20 % and 2 %) and time points (7 and 14 days), but no significant difference (p>0.05) in their deposition was observed between the MMC and the non-MMC counterparts at 20 % and 2 % oxygen tension and both time points (7 and 14 days).

Total protein amount in the cell layer was also quantified (Figure 3.10) and it was observed that at day 14 there was a significant increase in protein content at 2 % oxygen tension, independently of the use of CR (p<0.001).

Total amount of collagen was quantified using the Sircol™ Collagen Assay kit. Due to variability of the data, collagen content was presented as percentage of the total protein content. Statistical analysis revealed significant changes when CR was used independently of the oxygen tension (Figure 3.11; p<0.05).
To determine whether low oxygen tension and/or MMC influence the multipotent phenotype of BMSCs was assessed using tri-lineage differentiation assays (Figure 3.12). Adipogenic differentiation was significantly reduced ($p<0.001$) in low oxygen tension cultures, independently of the presence or absence of CR (Figure 3.12A). All treatments exhibited similar levels ($p>0.05$) of osteogenic differentiation (Figure 3.12B). Chondrogenic differentiation was not suppressed as a function of oxygen tension and MMC, with MMC treatments showing significantly higher ($p<0.001$) glycosaminoglycan (GAG) content at both 20 % and 2 % oxygen tension (Figure 3.12C).

Cell viability (Figure 3.13) was not affected ($p>0.05$) as a function of oxygen tension (20 % and 2 %) and presence or absence of CR.
Figure 3.1: (A) SDS PAGE showing collagen I deposition under various concentrations of carrageenan (1 to 500 µg/ml), non-crowded control (CTR) and a Ficoll™ cocktail (F: 37.5 mg/ml of Ficoll™ 70 kDa and 25 mg/ml of Ficoll™ 400 kDa) at 2, 4, 7 and 14 days and (B) corresponding densitometric quantification of α1(I) bands.
**Figure 3.2**: Immunocytochemistry images showing deposition of collagen I under various concentrations of carrageenan (1 to 500 µg/ml), non-crowded control (CTR) and a Ficoll™ cocktail (F; 37.5 mg/ml of Ficoll™ 70 kDa and 25 mg/ml of Ficoll™ 400 kDa) at 2, 4, 7 and 14 days.
Figure 3.3: Immunocytochemistry images showing deposition of collagen III under various concentrations of carrageenan (1 to 500 µg/ml), non-crowded control (CTR) and a Ficoll™ cocktail (F; 37.5 mg/ml of Ficoll™ 70 kDa and 25 mg/ml of Ficoll™ 400 kDa) at 2, 4, 7 and 14 days.
Figure 3.4: Immunocytochemistry images showing deposition of fibronectin under various concentrations of carrageenan (1 to 500 µg/ml), non-crowded control (CTR) and a Ficoll™ cocktail (F; 37.5 mg/ml of Ficoll™ 70 kDa and 25 mg/ml of Ficoll™ 400 kDa) at 2, 4, 7 and 14 days.
Figure 3.5: Immunocytochemistry images showing deposition of laminin under various concentrations of carrageenan (1 to 500 µg/ml), non-crowded control (CTR) and a Ficoll™ cocktail (F; 37.5 mg/ml of Ficoll™ 70 kDa and 25 mg/ml of Ficoll™ 400 kDa) at 2, 4, 7 and 14 days.
Figure 3.6: Cell viability of BMSCs under various concentrations of carrageenan (1 to 500 $\mu$g/ml), non-crowded control (CTR) and a Ficoll™ cocktail (F; 37.5 mg/ml of Ficoll™ 70 kDa and 25 mg/ml of Ficoll™ 400 kDa) at 2, 4, 7 and 14 days.
Figure 3.7: (A) SDS PAGE showing collagen I deposition under 20 % and 2 % oxygen tension, with (CR) and without carrageenan (CR) at 7 and 14 days and (B) corresponding densitometric quantification of α1(I) bands.
Figure 3.8: Immunocytochemistry images showing deposition of collagen I (A) and collagen III (B) under 20 % and 2 % oxygen tension, with (CR) and without carrageenan (CR) at 7 and 14 days.
Figure 3.9: Immunocytochemistry images showing deposition of fibronectin (A) and laminin (B) under 20 % and 2 % oxygen tension, with (CR) and without carrageenan (CR) at 7 and 14 days.
Figure 3.10: Total protein content deposited under 20 % and 2 % oxygen tension, with (CR) and without carrageenan (CR) at 7 and 14 days.
Figure 3.11: Total collagen content as percentage of total protein present in the cell layers deposited under 20 % and 2 % oxygen tension, with (CR) and without carrageenan (CR) at 7 and 14 days.
Figure 3.12: Trilineage differentiation of BMSCs\(^2\) after exposure to 20 % and 2 % oxygen tension, with (CR) and without carrageenan (CR) for 14 days showing (A) adipogenic differentiation and quantification of Oil Red O uptake, (B) osteogenic differentiation and quantification of Alizarin Red uptake and (C) chondrogenic differentiation and quantification of GAG content.

\(^2\)Adipogenic and osteogenic differentiation, Oil Red O and Alizarin Red S uptake quantification and GAG content quantification were performed by Daniela Cigognini.
Figure 3.13: Cell viability of BMSCs under 20 % and 2 % oxygen tension, with (CR) and without carrageenan (CR) at 7 and 14 days.
3.4 Discussion

Permanently differentiated and stem cell phenotype maintenance during *in vitro* expansion is at the forefront of scientific research and technological innovation for clinical translation and commercialisation of cell-based therapies. Although significant strides have been achieved using biophysical (e.g. surface topography, substrate rigidity and mechanical loading), biochemical (e.g. media supplements) and biological (e.g. growth factors) signals, the vast number of permutations on, for example, biochemical and / or biological media additives, concentrations, combinations and timings, has restricted the development of clinically relevant and commercially viable therapies. Further, none of these technologies enhances ECM deposition, resulting in prolonged cultures for the development of implantable devices based on the principles of modular tissue engineering, which are often associated with phenotypic drift and loss of cells’ therapeutic potential. Herein, we ventured to assess the simultaneous effect of oxygen tension and MMC on BMSCs ECM deposition and phenotype maintenance. The rationale of this approach is based on the simplicity in the implementation of the individual elements. SDS-PAGE and immunocytochemistry analyses clearly demonstrated an enhanced ECM deposition under MMC conditions, which was dependent on the crowder present (CR or F) and CR concentration. In dilute culture media, the de novo synthesised water soluble pro-collagen is dissolved before its N- and C- propeptide extensions are cleaved by the respective proteinases. The addition of inert macromolecules in the culture media restricts pro-collagen and proteinases diffusion, resulting in almost instantaneous pro-peptide extension cleavage and subsequent enhanced ECM deposition [31-33]. The 100 μg/ml CR concentration appeared to be the most effective in inducing maximum ECM deposition. Considering the theory of excluding volume effect, low concentrations of CR allow diffusion of pro-collagen / proteinases in the culture media, thus limiting ECM deposition. The superiority of CR over F in maximising ECM deposition is attributed to the inherent polydispersity of CR that most effectively excludes volume, as we have demonstrated previously [22].

Continuing with the simultaneous effect of low oxygen tension and MMC, we observed that MMC once more was effective in increasing ECM deposition. Previous studies indicate that enhanced HIF-1α expression under low oxygen tension leads to increased collagen synthesis through enhanced activity of collagen
However, as evidenced by SDS-PAGE and immunocytochemistry analysis, the use of hypoxia was not accompanied by enhanced ECM synthesis and subsequent deposition. These data are in accordance with previous studies, where adipose derived stem cells grown under low oxygen tension showed reduced ECM synthesis and cytokine type II secretion [35]. Nevertheless, it contradict previous studies with differentiated human embryonic stem cells [36], permanently differentiated cells (e.g. chondrocytes [37, 38], dermal fibroblasts [19, 39], renal epithelial cells [16], corneal fibroblasts [40] and naïve stem cells [41, 42], where enhanced ECM synthesis was observed under low oxygen tension conditions. It is interesting to also note the different impact of low oxygen tension on cells from different regions of intervertebral disc: low oxygen tension increased ECM synthesis in nucleus pulposus cells, whilst low oxygen tension did not bring about any significant difference in ECM synthesis in annulus fibrosus cells [43]. Collectively, these data suggest that activation of HIF-1α alone does not necessarily mean increased ECM synthesis; cell-specific endogenous factors (e.g. species; donor age and/or gender; tissue origin; cell differentiation stage) are crucial regulators of the influence of low oxygen tension on ECM synthesis. It is important to note that, under normoxia, the α subunit of HIF is targeted for degradation through O$_2$-dependent hydroxylation performed by prolyl hydroxylases [44]. However, in hypoxic conditions, the activity of the prolyl hydroxylases is affected, since O$_2$ is one of their co-factors, which hinders HIF hydroxylation, contributing to its activation [45, 46]. Considering that prolyl-4-hydroxylase is essential for the folding and stabilisation of the collagen triple helix during post-translational modifications, it is likely that hypoxic conditions can also impair the collagen biosynthesis process [47]. It is evidenced that further studies are required to elucidate the mechanism underlying the effect of HIF-1α, HIF-2α and other factors in ECM synthesis of permanently differentiated and stem cell populations. Nonetheless, the data demonstrate that tissue-engineering approaches based on low oxygen tension can be adapted to incorporate MMC without compromising ECM synthesis. Although it has been well-established in the literature that low oxygen tension (2 % to 8 %) is a critical factor in maintaining stem cell phenotype, function and self-renewal ex vivo [11, 48, 49], customarily in vitro cell expansion erroneously takes place at hyperoxic and deleterious for the cells high oxygen tensions (18 % to 20 %), which are often associated with
oxidative stress, DNA damage, growth arrest and loss of cells’ native phenotype and function [50-52].

Under low oxygen tension, protein synthesis has been described to be reduced as it is a very energy-demanding process for the cell that needs to adopt first to the new oxygen environment. Nonetheless, the Pierce BCA™ protein assay showed an increase, which might be associated with inherent limitations of the assay (e.g. interactions with ascorbic acid and phenol red, both present in the culture medium). Data collected using the Sircol™ assay, after normalisation to the total protein content, revealed that the total amount of collagen was increased when MMC was used. Similarly, for the Sircol™ Collagen assay, previous literature has raised concerns regarding the inaccuracy of this method [53], considering that the Sirius red dye used can bind to components in the FBS, such as BSA, which leads to an overestimation of the collagen content in cell culture samples [54]. Moreover, its use to stain histological sections is also non-specific to collagen fibres, since the dye also binds to basic amino acids in other proteins [55].

Osteogenic differentiation was not affected as a function of CR and oxygen tension and low oxygen tension reduced adipogenesis, as evidenced by the presence of less lipid vacuoles. These observations are in agreement with previous studies, where low oxygen tension maintained osteogenic differentiation [56] and supressed adipogenesis in a HIF-1α dependent manner [57]. However, it has also been shown that cells grown under low oxygen tension but differentiated under normoxia, can still successfully differentiate towards the adipogenic lineage [58]. These results also contradict previous literature, where osteogenesis was either promoted [59] or supressed [60] in low oxygen tension cultures due to downregulation of BMP2 and RUNX2 expression [61, 62], via IGFBP3 up-regulation [63] and inhibition of metabolic switch and mitochondrial function [64]. Moreover, hypoxia (5 % oxygen tension) in combination with low serum content (5 % FBS) has also been shown to enhance osteogenesis, by increasing ALP activity and matrix mineralisation [65]. It is also worth noting that extreme low oxygen tension (0.2 %) impaired osteogenic differentiation and enhanced adipogenic differentiation through over-expression of HIF-1α and CCAAT enhancer-binding proteins [66]. Further, although CR increased GAG content in the pellets, chondrogenesis was not affected as a function of low oxygen tension, which contradicts previous observations, where chondrogenic differentiation was promoted via activation of SOX-9, again, in HIF-
It appears that stem cell phenotype maintenance and/or differentiation as a function of oxygen tension, similarly to ECM synthesis as a function of oxygen tension, is dependent on numerous factors (e.g. species; donor age and/or gender; tissue origin; cell differentiation stage; media supplements; experimental conditions), imposing the need for standardisation.
3.5 Conclusions
Deposition of extracellular matrix by BMSCs was dependent on the crowder used, with CR outperforming F due to its inherent polydispersity and efficient exclusion of volume, also a consequence of its strong negative charge. Optimal ECM deposition was observed at 100 $\mu$g/ml of CR. Furthermore, hypoxia did not enhance collagen synthesis, as no differences were found in ECM deposition at 2 and 20 % oxygen tension. Surface marker expression of BMSCs was not affected by using CR or low oxygen tension, indicating maintenance of multipotent phenotype. In terms of differentiation potential, adipogenesis was reduced after cells were pre-exposed to low oxygen tension, while chondrogenesis was enhanced by pre-treatment with CR and osteogenesis remained unaffected.
3.6 References


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Chapter 4 - Tenogenic phenotype maintenance and trans-differentiation / differentiation using mechanical loading and macromolecular crowding

Sections of this chapter have been submitted for publication:

The influence of mechanical loading and macromolecular crowding on cell phenotype maintenance, trans-differentiation and differentiation, D. Gaspar, C. N. M. Ryan, D. Zeugolis, Submitted.
4.1 Introduction

Current tissue engineering and regenerative medicine strategies tested pre-clinically and clinically for tendon repair are mostly focused on direct cell injections [1-5], which are frequently associated with poor cell localisation at the site of injury [6, 7]. To enhance cell localisation, exogenous carriers are frequently used; however, they are often associated with foreign body / immune response [8, 9]. To overcome these limitations, tissue engineering by self-assembly has emerged [10]; the cell-produced and deposited ECM not only enables cell localisation at the site of injury, but also creates a physiologically relevant carrier. This approach has already been used successfully in clinical setting for skin [11], cornea [12] and blood vessel [13] tissue engineering. However, the large cell number and prolonged culture time required to produce an implantable construct are conducive to phenotypic drift and ultimately loss of cells’ therapeutic potential [14, 15]. Thus, it is imperative to develop strategies that will control cell fate during *in vitro* expansion and enhance ECM deposition, in a clinically relevant manner. Considering the complexity of native tissues, it has been recognised that monodomain approaches are unlikely to lead in a functional therapy; as such, multifactorial approaches are rapidly gaining pace in the field of tissue engineering [16-18].

In the tendon context, various *in vitro* microenvironment modulators (e.g. mechanical stimulation, topography, stiffness, oxygen tension, media supplementation, co-culture) are used to mimic the native tissue milieu, aiming to either maintain the phenotype of tenocytes and tendon stem cells or to direct other cell types (e.g. dermal fibroblasts, muscle-derived cells, bone marrow derived mesenchymal stem cells, adipose derived stem cells) towards the tenogenic lineage [19, 20]. Given that the function of tendon is to transmit mechanical forces between muscle and bone, mechanical stimulation has been used extensively in a static and dynamic manner as means to induce tenogenic phenotype [21-26]. The use of uniaxial dynamic loading regimens is known to maintain physiological tenocyte morphology, which topography alone is not always adequate to do so [27], and to induce increased synthesis of collagen types I and III, the main components of tendon ECM, and expression of tenascin-C, tenomodulin and scleraxis, which are commonly regarded as tenogenic markers [28-30].

However, mechanical loading alone, only marginally increases ECM deposition. This is not surprising considering that in the very dilute culture conditions currently
used, the *de novo* synthesised ECM is discarded during media changes. Recent data have demonstrated that macromolecular crowding (MMC), the addition of inert and polydisperse macromolecules (e.g. carrageenan, dextran sulphate, Ficoll™ cocktail of 70 and 400 kDa) to the culture environment, significantly accelerates ECM deposition [31, 32]. It is worth noting that carrageenan significantly increases ECM deposition [33-36], without, however, affecting COL1A1 and prolyl-4-hydroxylase gene expression in bone marrow stem cells and corneal fibroblasts [32]. This enhanced ECM deposition is due to the volume excluded by these macromolecules that affects the rate of biological processes [37, 38]. In terms of ECM deposition, MMC creates a confined and dense setting that accelerates the enzymatic conversion of procollagen to collagen, which is a very slow process in the dilute culture media conditions [39-41]. Thus far, MMC has been shown to enhance ECM deposition in various permanently differentiated and stem cell populations [31, 32]. Although MMC has been previously combined with other microenvironmental modulators, such as hypoxia [42, 43], it is yet to be used simultaneously with mechanical stimulation. Herein, we ventured to assess the synergistic effect of mechanical stimulation and MMC on cell [human adult tenocytes (TCs), human neonatal dermal fibroblasts (NDFs), human adult dermal fibroblasts (ADFs) and human bone marrow stem cells (BMSCs)] morphology, deposited ECM composition and cell phenotype maintenance (TCs), differentiation (BMSCs) and trans-differentiation (NDFs and ADFs).
4.2 Material and Methods

4.2.1 Materials
Tissue culture consumables were purchased from Sarstedt (Ireland) and NUNC (Denmark). All chemicals, cell culture media and reagents were purchased from Sigma Aldrich (Ireland), unless otherwise stated.

4.2.2 Cell culture, macromolecular crowding
Human tenocytes (TCs) and human adult (ADFs) and human neonatal (NDFs) dermal fibroblasts were purchased from DV Biologics (USA) and LG Standards (UK), respectively. Human bone marrow mesenchymal stem cells (BMSCs) were isolated from fresh bone marrow (Lonza, UK), as described previously [39]. TCs, ADFs and NDFs were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10 % foetal bovine serum (FBS) and 1 % penicillin streptomycin (PS). BMSCs were cultured in alpha Minimum Essential Medium with GlutaMax™ (Gibco Life Technologies, UK), supplemented with 10 % FBS and 1 % PS. Cells were seeded at 25,000 cells / cm² and allowed to attach for 24 hours before starting uniaxial stretching and changing the medium to MMC medium containing 100 µM of L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate and 100 µg/ml of carrageenan (CR).

4.2.3 Mechanical loading
Mechanical stimulation was applied using a Mechanoculture FX system from CellScale (Canada). This a uniaxial loading system for cell culture that supports flexible silicone 24 well plates. Cells were seeded directly onto the silicone wells without any surface coating and allowed to attach for 24 hours before initiating mechanical stimulation. The loading regime applied consisted of a 10 % substrate strain (silicone well plate was stretched uniaxially to 10% of its initial length) at a frequency of 1 Hz (0.5 seconds for stretching and 0.5 seconds for recovery without holding position) continuously for 12 hours per day. As recommended by the manufacturer, 2 horizontal slits were created alongside the outside limits of the well plate to ensure strain uniformity in all wells.
4.2.4 Cell morphology
At each time point (3 and 7 days), cells were fixed with 2 % paraformaldehyde (PFA), permeabilised with 0.2 % Triton X-100 and stained with fluorescein isothiocyanate (FITC) labelled phalloidin to mark the cytoskeleton and 4',6-diamidino-2-phenylindole (DAPI) for the nucleus. Samples were imaged in an inverted fluorescence microscope (Olympus IX81, Olympus, Japan). Morphometric analysis for quantification of orientation, area and aspect ratio was performed using ImageJ (NIH, USA).

4.2.5 Immunocytochemistry
At each time point, cells were briefly washed with Hank’s Balanced Salt Solution (HBSS) and fixed with 2 % PFA for 15 minutes at room temperature. Cell were washed again and non-specific site were blocked with 3 % bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 30 minutes. Cells were then incubated for 90 minutes at room temperature with the primary antibodies (Abcam, UK) for collagen types I (ab90935), III (ab7778), IV (ab6586), V (ab7046) and VI (ab6588), after which were washed three times with PBS, followed by 30 minutes of incubation at room temperature with the secondary antibody (Alexa Fluor® 488 goat anti-rabbit A11034 and Alexa Fluor® 488 goat anti-mouse A11001, Life Technologies, UK). Nuclei were counterstained with DAPI. Fluorescent images were captured with an inverted fluorescence microscope (Olympus IX-81, Olympus Japan) and images were further processed with ImageJ software (NIH, USA). Nuclei were counted to obtain cell number per area at the different time points and fluorescent area per image was quantified.

4.2.6 Gene expression
Gene expression analysis was conducted using a RealTime ready Custom Panel for RT-qPCR (Roche, Ireland) to assess expression of tenogenic [collagen type I (COL1A1), scleraxis (SCXA), tenomodulin (TNMD), tenascin-C (TNC), thrombospondin 4 (THBS4)], chondrogenic [collagen types II (COL2A1) and X (COL10A1), aggrecan (ACAN), cartilage oligomeric matrix protein (COMP)] and osteogenic [runt-related transcription factor 2 (RUNX2), secreted protein acidic and rich in cysteine (SPARC), alkaline phosphatase (ALPP), integrin binding sialoprotein (IBSP)] markers. Briefly, total RNA was extracted from the different
cell sources at days 3 and 7 using TRI Reagent® for 5 minutes at room temperature to lyse the cells. The Tri Reagent® was collected, chloroform was added and the solution was vortexed for 15 seconds and incubated at room temperature for 5 minutes. The solution was centrifuged and the upper aqueous phase containing the RNA was collected and mixed with ethanol, after which the solution was purified using the High Pure RNA isolation kit (Roche, Ireland). Total RNA concentration and quality were analysed using the NanoDrop 1000 (Thermo Scientific, Ireland) and the Agilent 2100 Bioanalyser (Agilent Technologies, Ireland). Samples with RNA integrity number above 8 and with A260 / A280 between 2 and 2.2 were used for further experiments. RNA was transcribed to cDNA using a Transcriptor First Strand cDNA synthesis kit (Roche, Ireland) and 1 µg of RNA sample was used throughout all groups. The thermal block cycle was as follows: 1 hour at 50 ºC and 5 minutes at 85 ºC for enzyme inactivation. 1 µl of transcribed cDNA was added to 9 µl of probes master into a RealTime ready custom 384 well plate (Roche, Ireland). Negative controls of empty wells and untranscribed RNA were utilised and plate was run in the LightCycler® 480 Instrument (Roche, Ireland). For analysis, genes were normalised to the housekeeper gene (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) and fold change was obtained using the $2^{-\Delta\Delta Ct}$ as previously described [46]. Z-scores of fold changes were calculated and relevant up- and downregulations were accepted when score was at least two standard deviations away from the mean value of fold-change for each gene.

4.2.7 Cell viability

4.2.7.1 Cytotox 96® Non-radioactive Cytotoxicity Assay
This method was utilised during optimisation of mechanical loading regime. Assay was performed following manufacturer’s instructions. Briefly, culture medium was collected, the reconstituted Substrate Mix was added to collected medium and incubated at room temperature for 30 minutes. Absorbance was then read at 490 nm. Samples were compared with a standard curve of different dilutions of lysed cells and percentage of cell viability was calculated based on the amount of lactate dehydrogenase present.
4.2.7.2 Live / dead staining
At each time point, a calcein AM and ethidium homodimer I stainings were performed to assess the influence of the MMC and mechanical stimulation on cell viability, as per manufacturer’s protocol. Briefly, cells were washed with HBSS and a solution of calcein AM (4 µM) and ethidium homodimer I (2 µM) was added. Cells were incubated at 37 ºC and 5 % CO₂ for 30 minutes after which fluorescent images were acquired with an Olympus IX-81 inverted fluorescence microscope (Olympus, Tokyo, Japan).

4.2.8 Cell metabolic activity
At each time point, the alamarBlue® assay was used to evaluate the influence of mechanical stimulation and MMC on cell metabolic activity, as per manufacturer’s instructions. Briefly, at each time point, cells were washed with HBSS and a 10 % alamarBlue® solution in HBSS was added to the cells. Cells were incubated at 37 ºC and 5 % CO₂ for 3 hours and absorbance was measured at 550 nm and 595 nm with a Varioskan Flash Spectral scanning multimode reader (Thermo Scientific). Cell metabolic activity was expressed on terms of percentage reduction of the alamarBlue® dye and normalised to the non-crowded static control.

4.2.9 Statistical analysis
Data is expressed as mean ± standard deviation. Statistical analysis was performed using MINITAB version 16 (Minitab Inc., USA). One- or two-way analysis of variance (ANOVA) was used for multiple comparisons and a Tukey post hoc test was used for pairwise comparisons after confirming the samples followed a normal distribution (Kolmogorov-Smirnov test) and had equal variances (Bartlett’s and Levene’s test for homogeneity of variances). When one of these assumptions was violated, non-parametric tests were used for multiple comparisons (Kruskall-Wallis test) and pairwise comparisons (Mann-Whitney test). Statistical significance was accepted at \( p<0.05 \)
4.3 Results

4.3.1 Optimisation of mechanical loading conditions

Uniaxial strain was optimised using TCs and ADFs. An initial load of 4 % strain was applied for 8 hours per day to mimic physiological movement. This loading regime was applied to TCs for 3 days, after which it was observed that TC morphology (Figure 4.1A) and orientation (Figure 4.1B) were not altered by mechanical stimulation either in the presence or absence of MMC. Moreover, cell metabolic activity (Figure 4.1B), viability (Figure 4.1D) and proliferation (Figure 4.1E) were also not significantly altered. ADFs were exposed to the same mechanical loading conditions and, similarly to the TCs, morphology (Figure 4.2A) and orientation of cytoskeleton (Figure 4.2B) were not altered with regards to the uniaxial load utilised and cell metabolic activity (Figure 4.2C), viability (Figure 4.2D) and proliferation (Figure 4.2E) were also not negatively affected by dynamic loading or MMC.

To achieve preferential cell orientation under mechanical stimulation, a uniaxial strain of 10 % was applied to ADFs for 6, 12 and 24 hours to determine the necessary amount of loading time to impact cell morphology. At 6 hours, ADFs were still not fully spread on the substrate. Nonetheless, at 12 hours, cytoskeleton orientation perpendicular to the load was observed, and maintained throughout for 24 hours (Figure 4.3). Morphometric analysis at the different time points confirmed that at 12 hours at least 60 % of the cell population showed orientation perpendicular to the load (Figure 4.4). Aspect ratio of cell cytoskeleton was found to increase significantly for all groups over time due to formation of attachment points after 6 hours of mechanical stimulation ($p<0.001$; Figure 4.5A). Cell area also presented significant variations under mechanical loading (Figure 4.5B). MMC did not affect cell morphology or orientation. Considering these results, a dynamic loading regime of 10 % strain for 12 hours per day was utilised for all subsequent experiments.
Chapter 4

Figure 4.1: Tenocytes cultured for 3 days under uniaxial stretching (4 % strain) for 8 hours per day. (A) Cell morphology did not show preferential alignment with regards to uniaxial loading (white arrow indicates direction of mechanical load) as confirmed by morphometric analysis (B). (C) Cell metabolic activity, (D) cell viability and (E) cell proliferation were not significantly altered by the presence of MMC or dynamic loading.
Figure 4.2: ADFs cultured for 3 days under uniaxial stretching (4 % strain) for 8 hours per day. (A) Cell morphology does not show preferential alignment with regards to uniaxial loading (white arrow indicates direction of mechanical load) as confirmed by morphometric analysis (B). (C) Cell metabolic activity, (D) cell viability and (E) cell proliferation were not significantly altered by the presence of MMC or dynamic loading.
Figure 4.3: Cell morphology of ADFs cultured under uniaxial stretching (10% strain) for 6, 12 and 24 hours. Cells exhibit alignment perpendicular to the applied load at 12 and 24 hours. White arrow indicates direction of mechanical load.
Figure 4.4: Morphometric analysis showing distribution of orientation of the cell cytoskeleton of ADFs cultured under uniaxial stretching (10 % strain) for 6 (A), 12 (B) and 24 hours (C). Cells aligned perpendicularly to the applied load after 12 and 24 hours of continuous loading.
Figure 4.5: Morphometric analysis of cell cytoskeleton of ADFs cultured under uniaxial stretching (10 % strain) for 6, 12 and 24 hours. (A) Aspect ratio was found to increased significantly over time for all groups due to initial cell spreading to establish surface attachment points ($p<0.001$). (B) Cell area varied significantly over time for both groups under mechanical loading (10 % -MMC and 10 % +MMC) ($p<0.05$).
4.3.2 Cellular morphology and morphometric analysis

Cellular morphology was evaluated after exposure to mechanical loading and MMC for 3 and 7 days. TCs presented cytoskeleton alignment perpendicular to the applied load at days 3 and 7 (Figure 4.6A) and MMC did not affect cell orientation. Morphometric analysis revealed that between 40 and 70 % of cells exhibited preferential alignment at days 3 (Figure 4.7A) and 7 (Figure 4.7B). Moreover, the cytoskeleton area (Figure 4.7C) and the aspect ratio (Figure 4.7D) were not significantly ($p>0.05$) affected. The cytoskeleton of NDFs (Figure 4.6B) and ADFs (Figure 4.6C) also demonstrated perpendicular alignment to the load at days 3 and 7. Morphometric analysis confirmed the preferential orientation and revealed an increase in percentage of cells aligned from day 3 (Figure 4.8A for the NDFs and Figure 4.9A for the ADFs) to day 7 (Figure 4.8B for the NDFs and Figure 4.9B for the ADFs). Area (Figure 4.8C for the NDFs and Figure 4.9C for the ADFs) and aspect ratio (Figure 4.8D for the NDFs and Figure 4.9D for the ADFs) of cytoskeleton also increased significantly ($p<0.05$) under mechanical loading. At day 3, the morphology of BMSCs was not affected by mechanical stimulation or MMC (Figure 4.6D), while at day 7, some alignment perpendicular to the load was observed. Morphometric analysis confirmed the random cell orientation seen at day 3 (Figure 4.10A) and revealed that around 40 % of cells aligned perpendicularly at day 7 (Figure 4.10B). Further, area (Figure 4.10C) and aspect ratio (Figure 4.10D) were not significantly ($p>0.05$) affected by uniaxial loading or MMC. Nuclei orientation assessment revealed two peaks in the vicinity of 90°, when 10 % strain was used at day 3 for the permanently differentiated cells (Figure 4.11A for TCs, Figure 4.12A for NDFs and Figure 4.13A for ADFs), but not for the BMSCs (Figure 4.14A), whilst, at day 7, this trend was observed with all cell types ((Figure 4.11B for TCs, Figure 4.12B for NDFs, Figure 4.13B for ADFs and Figure 4.14B for BMSCs). TCs presented a significant increase in nuclei area at day 7 at 0% strain (Figure 4.11C; $p<0.001$). NDFs (Figure 4.12C) and ADFs (Figure 4.13C) exhibited significant changes in nuclei area under mechanical stimulation at both time points ($p<0.02$), whilst BMSCs presented an increase in all groups from day 3 to day 7 (Figure 4.14C; $p<0.001$). Nuclei aspect ratio of TCs did not change with mechanical stimulation or MMC (Figure 4.11D; $p>0.05$), NDFs showed a significant decrease at day 7 at 0 % strain with MMC (Figure 4.12D; $p<0.05$), ADFs exhibited an increase also at day 7 at 0 % strain without MMC (Figure
4.13D; \( p < 0.05 \) and BMSCs displayed a significant increase at day 7 at 10 \% strain (Figure 4.14D; \( p < 0.001 \)).
Figure 4.6: Cytoskeleton and nuclei staining of TCs (A), NDFs (B), ADFs (C) and BMSCs (D) at 3 and 7 days of culture in the absence (-MMC) and presence (+MMC) of macromolecular crowding and static (0 %) and dynamic (10 %) culture conditions. White arrow indicates direction of applied load. Strict perpendicular alignment to the load was observed with the permanently differentiated cells.
Figure 4.7: Morphometric analysis of the cytoskeleton of TCs in the absence (-MMC) and presence (+MMC) of macromolecular crowding and static (0 %) and dynamic (10 %) culture conditions. Quantification of cell alignment at day 3 (A) and day 7 (B) revealing perpendicular alignment to the load under 10 % strain. Cytoskeleton area (C) and aspect ratio (D) were not significantly affected by MMC or load applied ($p>0.05$).
Figure 4.8: Morphometric analysis of the cytoskeleton of NDFs in the absence (-MMC) and presence (+MMC) of macromolecular crowding and static (0 %) and dynamic (10 %) culture conditions. Quantification of cell alignment at day 3 (A) and day 7 (B) revealing perpendicular alignment to the load under 10 % strain. Cytoskeleton area (C) and aspect ratio (D) were significantly affected by 10% strain ($p<0.05$).
Figure 4.9: Morphometric analysis of the cytoskeleton of ADFs in the absence (-MMC) and presence (+MMC) of macromolecular crowding and static (0 %) and dynamic (10 %) culture conditions. Quantification of cell alignment at day 3 (A) and day 7 (B) revealing perpendicular alignment to the load under 10 % strain. Cytoskeleton area (C) and aspect ratio (D) were significantly affected by 10 % strain ($p<0.05$).
Figure 4.10: Morphometric analysis of the cytoskeleton of BMSCs in the absence (-MMC) and presence (+MMC) of macromolecular crowding and static (0 %) and dynamic (10 %) culture conditions. Quantification of cell alignment at day 3 (A) and day 7 (B) revealing little preferential under 10 % strain. Cytoskeleton area (C) and aspect ratio (D) were not significantly affected by MMC or load applied ($p>0.05$).
Figure 4.11: Morphometric analysis of the nuclei of TCs in the absence (-MMC) and presence (+MMC) of macromolecular crowding and static (0 %) and dynamic (10 %) culture conditions. Nuclei alignment at days 3 (A) and 7 (B) did not reveal preferential orientation, whilst nuclei area (C) was significantly increased at day 7 at 0% strain ($p<0.001$) and aspect ratio was unchanged (D).
Figure 4.12: Morphometric analysis of the nuclei of NDFs in the absence (-MMC) and presence (+MMC) of macromolecular crowding and static (0 %) and dynamic (10 %) culture conditions. Nuclei alignment at days 3 (A) and 7 (B) did not reveal preferential orientation, whilst nuclei area (C) presented significant changes at 10 % strain ($p<0.02$) and aspect ratio (D) was decreased at day 7 at 0% strain with MMC ($p<0.05$).
Figure 4.13: Morphometric analysis of the nuclei of ADFs in the absence (-MMC) and presence (+MMC) of macromolecular crowding and static (0 %) and dynamic (10 %) culture conditions. Nuclei alignment at days 3 (A) and 7 (B) did not reveal preferential orientation, whilst nuclei area (C) presented significant changes at 10 % strain ($p<0.02$) and aspect ratio (D) was increased at day 7 at 10% strain with MMC ($p<0.001$).
Figure 4.14: Morphometric analysis of the nuclei of BMSCs in the absence (-MMC) and presence (+MMC) of macromolecular crowding and static (0 %) and dynamic (10 %) culture conditions. Nuclei alignment at days 3 (A) and 7 (B) did not reveal preferential orientation, whilst nuclei area (C) increased from day 3 to day 7 in all groups ($p<0.001$) and aspect ratio (D) increased at day 7 under 10 % strain.
4.3.3 Extracellular matrix composition and orientation

Immunocytochemistry and corresponding quantification of fluorescent area were performed at days 3 and 7 for all different cell sources. TCs showed significantly increased deposition of collagen type I at day 3, when mechanical loading was used in combination with MMC (Figure 4.15A) ($p<0.05$). At day 7, in TC cultures, collagen type I was detected under static and dynamic conditions, when MMC was used ($p<0.05$). NDFs (Figure 4.15B) and ADFs (Figure 4.15C) presented increased deposition of collagen type I under MMC and independently of loading conditions at both time points ($p<0.01$). BMSCs (Figure 4.15D) also revealed significantly enhanced deposition at day 3 under MMC conditions, whilst at day 7, collagen type I was increased under uniaxial loading and MMC conditions ($p<0.01$).

TCs (Figure 4.16A) presented significantly increased deposition of collagen type III at day 3 under MMC, while at day 7, mechanical stimulation induced a decrease ($p<0.001$). Collagen type III was deposited by NDFs at all time points and no differences were found between the different groups (Figure 4.16B). At day 3, ADFs (Figure 4.16C) presented a decrease in collagen type III in mechanically stimulated groups ($p<0.001$), whilst at day 7 no significant changes were found. BMSCs (Figure 4.16D) did not exhibit significant variations in collagen type III deposition at day 3, whilst at day 7, a decrease was observed under 10 % strain ($p<0.01$).

TCs (Figure 4.17A) did not deposit collagen type IV at the examined time points. NDFs (Figure 4.17B) and ADFs (Figure 4.17C) presented deposition of small amounts, with no significant changes being observed due to loading or MMC ($p>0.05$). BMSCs deposited the highest amount of collagen IV (Figure 4.17D); no changes were observed at day 3, whilst at day 7, there was a significant decrease under MMC conditions ($p<0.01$).

TCs (Figure 4.18A) presented increased deposition of collagen type V under MMC at day 3, whilst an overall decrease was observed at day 7 ($p<0.01$). NDFs (Figure 4.18B) did not display any significant changes at day 3, whilst at day 7, both mechanical loading and MMC led to increased deposition ($p<0.05$). Similarly, ADFs (Figure 4.18C) did not exhibit significant variations at day 3, whereas at day 7, there was a significant decrease under mechanical stimulation in the absence of MMC ($p<0.001$). BMSCs revealed a significant increase of collagen type V at day...
when 10 % loading was combined with MMC and no differences were found at day 7 ($p<0.05$; Figure 4.18D).

TCs (Figure 4.19A) and NDFs (Figure 4.19B) presented a decrease in collagen type VI deposition under mechanical stimulation, which was compensated by the use of MMC ($p<0.05$). ADFs (Figure 4.19C) and BMSCs (Figure 4.19D) did not show significant changes ($p>0.05$).

Images of the different collagen types were used for morphometric analysis of ECM orientation. For all cell sources, ECM was orientated in the same direction of the cytoskeleton: permanently differentiated cells exhibited ECM alignment perpendicular to the load at both time points, whilst BMSCs only revealed a small percentage of preferential orientation at day 7 (Figure 4.20 for day 3 and Figure 4.21 for day 7).
Figure 4.15: Immunocytochemistry staining and corresponding fluorescent area quantification of collagen type I at days 3 and 7 in the absence (-MMC) and presence (+MMC) of macromolecular crowding under static (0 %) and dynamic (10 %) culture conditions. (A) TCs presented significantly increased deposition at day 3 at 10 % strain and MMC and, at day 7, collagen I was significantly increased when MMC was used independently of the use of load (p<0.05). (B) NDFs and (C) ADFs exhibited increased deposition when MMC was used (p<0.01). (D) BMSCs,
at day 3 displayed significantly enhanced deposition under MMC, while at day 7, a significant increase was seen at 10 % +MMC ($p<0.01$).
Figure 4.16: Immunocytochemistry staining and corresponding fluorescent area quantification of collagen type III at days 3 and 7 in the absence (-MMC) and presence (+MMC) of macromolecular crowding under static (0 %) and dynamic (10 %) culture conditions. (A) TCs presented significantly increased deposition at day 3 when MMC was used. However, at day 7 there was a significant decrease at 10 % strain ($p<0.001$). (B) NDFs deposited collagen III under all conditions. (C)
ADFs, at day 3, exhibited a significant decrease under 10 % ($p<0.001$), whilst no differences were found at day 7. (D) BMSCs presented a significant decrease at day 7 at 10 % strain ($p<0.01$).
Figure 4.17: Immunocytochemistry staining and corresponding fluorescent area quantification of collagen type IV at days 3 and 7 in the absence (-MMC) and presence (+MMC) of macromolecular crowding under static (0 %) and dynamic (10 %) culture conditions. (A) TCs did not present deposition of collagen type IV. (B) NDFs and (C) ADFs deposited small amounts of collagen IV yet no differences were found ($p>0.05$). (D) BMSCs exhibited a significant decrease under MMC at day 7 ($p<0.01$).
Figure 4.18: Immunocytochemistry staining and corresponding fluorescent area quantification of collagen type V at days 3 and 7 in the absence (-MMC) and presence (+MMC) of macromolecular crowding under static (0 %) and dynamic (10 %) culture conditions. (A) TCs presented significantly increased deposition under MMC at day 3 and an overall decrease at day 7 ($p<0.01$). (B) NDFs presented a significant increase with the use of MMC and 10 % strain ($p<0.05$). (C) ADFs, at day 7, displayed a significant decrease at 10 % strain without MMC ($p<0.001$). (D)
BMSCs exhibited a significant increase at day 3 under 10 % strain and MMC ($p<0.05$).
**Figure 4.19:** Immunocytochemistry staining and corresponding fluorescent area quantification of collagen type VI at days 3 and 7 in the absence (-MMC) and presence (+MMC) of macromolecular crowding under static (0 %) and dynamic (10 %) culture conditions. (A) TCs and (B) NDFs presented significantly increased deposition at 10 % strain in the presence of MMC ($p<0.05$). (C) ADFs and (D) BMSCs deposited collagen VI under all conditions.
Figure 4.20: ECM orientation of (A) tenocytes, (B) NDFs, (C) ADFs and (D) BMSCs at day 3 in the absence (-MMC) and presence (+MMC) of macromolecular crowding and under static (0 %) and dynamic (10 %) conditions.
Figure 4.21: ECM orientation of (A) tenocytes, (B) NDFs, (C) ADFs and (D) BMSCs at day 7 in the absence (-MMC) and presence (+MMC) of macromolecular crowding and under static (0 %) and dynamic (10 %) conditions.


4.3.4 Gene expression

Expression of different genes associated with tenogenic, chondrogenic and osteogenic phenotype were analysed to assess phenotype maintenance (TCs), differentiation (BMSCs) and trans-differentiation (NDFs and ADFs). TNMD and COL2A1 were late detections (as were all other genes / conditions represented in white) and, therefore were not quantifiable for any of the cell sources tested. IBSP was only expressed by TCs and BMSCs.

TC gene expression at day 3 was unchanged (Figure 4.22A), whilst at day 7, upregulation of SCXA at 10 % strain was observed. THBS4 and COMP were also upregulated at 10 %, but only in the absence of MMC.

At day 3, NDFs exhibited downregulation of RUNX2 under static conditions with MMC, whilst 10 % strain in the absence of MMC led to increased SCXA, THBS4 and ALPP (Figure 4.22B). At day 7, 0 % strain and MMC induced downregulation of ACAN, whilst mechanical stimulation without MMC led to decreased COL1A1 and increased COL10A1. The combination of 10 % strain and MMC induced upregulation of SCXA and ALPP.

ADFs (Figure 4.22C), at day 3, under 10 % strain exhibited upregulation of TNC, whereas adding MMC induced upregulation of ACAN and downregulation of COMP. At day 7, again at 10 % strain, in the absence of MMC, there was upregulation of SCXA, THBS4, COL10A1 and IBSP, while the use of MMC, only led to upregulation of THBS4 and COL10A1.

BMSCs (Figure 4.22D) exhibited upregulation of THBS4 and ALPP under static conditions and MMC at day 3. Further, at 10 % strain, downregulation of COL1A1 was observed, whilst in the presence of MMC, upregulation of ALPP was evidenced. At day 7, only upregulation of COL10A1 was observed at 10 % strain without MMC. At day 7, mechanical loading and MMC did not induce any changes in the gene expression profile of BMSCs.
Figure 4.22: Taqman® array showing expression of tenogenic, chondrogenic and osteogenic genes by TCs, NDFs, ADFs and BMSCs after 3 and 7 days of culture under static (0 %) and dynamic (10 %) conditions in the absence (-MMC) and presence (+MMC) of macromolecular crowding.
4.3.5 Cell viability, metabolic activity and proliferation
Neither MMC nor mechanical loading affected cell viability (Figure 4.23). Cell metabolic activity of TCs and BMSCs was significantly increased at day 7 under mechanical stimulation ($p<0.05$), whilst ADFs and NDFs did not exhibit significant variations (Figure 4.24). TCs presented a decrease in cell number at day 7 under MMC, while cell number of NDFs and BMSCs was decreased significantly at day 7 after mechanical stimulation ($p<0.05$); ADFs did not exhibit significant changes ($p>0.05$) in cell number (Figure 4.25).
Figure 4.23: Cell viability of (A) TCs, (B) NDFs, (C) ADFs and (D) BMSCs at day 7 in the absence (-MMC) and presence (+MMC) of macromolecular crowding and under static (0 %) and dynamic (10 %) conditions. Live cells are represented in green and dead cells in red.
Figure 4.24: Cell metabolic activity of (A) TCs, (B) NDFs, (C) ADFs and (D) BMSCs at day 3 and 7 in the absence (-MMC) and presence (+MMC) of macromolecular crowding and under static (0 %) and dynamic (10 %) conditions.
Figure 4.25: Cell proliferation of (A) TCs, (B) NDFs, (C) ADFs and (D) BMSCs at day 3 and 7 in the absence (-MMC) and presence (+MMC) of macromolecular crowding and under static (0 %) and dynamic (10 %) conditions.
4.4 Discussion

Tissue engineering strategies for tendon repair and regeneration rely heavily on the use of TCs, the native cell type of tendon tissue. However, these cells undergo phenotypic drift in vitro, which compromises their therapeutic potential [15]. Other cell sources have emerged as alternatives, but it is necessary to induce tenogenic differentiation / trans-differentiation to ensure functional specificity and secretion of tendon-like tissue. To maintain TC phenotype or to direct other cell types towards tenogenic lineage, microenvironmental cues (biophysical, biochemical and biological in origin) have been used to better imitate the complex tendon microenvironment. In this context, biophysical cues are particularly significant, since topographical and mechanical features are key characteristics of tendon tissues, whilst macromolecular crowding, by imitating the dense extracellular space, is relevant to any tissue engineering strategy. Herein, we ventured to evaluate the simultaneous effect of mechanical stimulation and MMC on cell morphology, deposited ECM composition and cell phenotype maintenance (TCs), differentiation (BMSCs) and trans-differentiation (NDFs and ADFs).

Initially, based on previous literature, a strain of 4 % at 1 Hz for 8 hours was used [21, 44-46]. TCs exposed to this loading regime did not exhibit preferential cytoskeletal alignment and similar behaviour was found with the ADFs. Since previous literature suggests that cell orientation is highly dependent on strain amplitude [47-49], strain was increased to 10 % and different loading times were tested. Morphometric analysis showed that, after 12 hours of loading, more than 60 % of cultured ADFs were aligned in the direction perpendicular to the load, and this was maintained after 24 hours of continuous loading. Therefore, a strain amplitude of 10 % for 12 hours per day was used for further experiments.

Cytoskeleton and nuclei staining and respective morphometric analysis revealed strict preferential alignment of the permanently differentiated cell sources (TCs, NDFs and ADFs) perpendicular to the applied load, whilst BMSCs did not show preferential alignment. Perpendicular orientation is concordant with previous reports where melanocytes [47], fibroblasts [48-51], endothelial [52-55] and smooth muscle cells [56, 57] presented similar behaviour. If perpendicular alignment is not desirable, contact guidance can be used in combination with mechanical stimulation, as cells grown in grooved or fibrous substrates align in the direction provided by the surface independently of the direction of the load [58-60].
It has been suggested that the perpendicular alignment occurs due to excessive cell length change: cells try to avoid high axial surface strains and relieve the tension increase in the stretch fibres by adopting a more stable configuration of the actin cytoskeleton [47, 49, 50, 54]. Area and aspect ratio of NDFs and ADFs was also significantly increased by mechanical stimulation, which is related with the higher alignment presented by these cell types. The area and aspect ratio of TCs and BMSCs was not significantly affected by mechanical stimulation and, even under static conditions the cytoskeleton of these cells presented a larger area than NDFs and ADFs. Considering that cell size depends on cytoskeletal filament organisation and number of established focal adhesions (the amount of focal adhesions increases proportionally with cell size [61, 62]), this indicates that TCs and BMSCs presented a more stable cytoskeleton configuration, due to the larger area and number of focal adhesions, thus not being as easily affected as the dermal fibroblasts. Nonetheless, the TCs exhibited preferential alignment, whilst the BMSCs did not, which demonstrates that the extent of morphological changes can provide information regarding the differentiation state of the cells. Previous work with grooved and fibrous topographies suggests that the cell alignment induced by the underlying surface features correlates with phenotypic changes [63-65]. Regardless, BMSCs results contradict previous reports where the of use of similar strain and frequency conditions induced perpendicular alignment to the load [66-68]. Further, it has been shown that these cells respond to mechanical stimulation and can adapt their phenotype to strain amplitude [69] and type of stimulation [70]. Furthermore, there is evidence that suggests that cells retain memory from their original tissue, which can make them behave differently in culture. This has been shown particularly in developmental studies or in induced pluripotent stem cells that present preferential differentiation towards their original lineage [71-73]. This could prompt differential behaviour in terms of cytoskeleton adaptation to mechanical stimulation, since TCs, ADFs and NDFs derive from tissues frequently exposed to strain while BMSCs are shielded from direct load application in the bone marrow. Overall it is important to consider that the strain applied to substrate is felt differently by the cells and it has been suggested that a substrate strain of 10 % corresponds to 3 % to 5 % of cell strain [74, 75]. Moreover, literature indicates that there is a cell-specific threshold strain below which cells do not reorient when stimulated, given that strain amplitude and necessary loading time are extremely variable [48, 53, 57, 76],
indicating that tailored loading regimes are needed to obtain specific responses from a cell source. Analysis of nuclei orientation did not reveal preferential alignment concordant with cytoskeleton orientation, while significant changes were detected in terms of nuclei aspect ratio and area. Nuclei shape changes have been shown to influence gene expression and protein synthesis and thereby direct cell phenotype [77-80]. However, it is often overlooked and more in-depth studies are needed to fully understand how modulating nuclei shape can help understanding phenotypic commitment. ECM alignment was observed to follow alignment of cell cytoskeleton for all cell sources. This is concordant with previous work with mechanical stimulation and aligned anisotropic topographies [81].

Regarding ECM composition, immunocytochemistry analysis revealed increased deposition of collagen type I, when MMC was used at both 0 and 10 % strain with NDFs, ADFs and BMSCs at days 3 and 7. TCs presented a similar behaviour at day 7, whilst at day 3, collagen type I deposition was only found when mechanical stimulation was combined with MMC. Enhanced deposition of collagen type I due to MMC has been described repeatedly in the literature for various crowders [32, 39-41, 82]. Among them, carrageenan has shown superior performance in terms of accelerated and enhanced ECM deposition by TCs, osteoblasts, BMSCs and dermal, lung and corneal fibroblasts [31, 32]. The confined environment created by the high natural polydispersity of carrageenan in the culture medium accelerates cleavage of the N- and C- propeptides of the procollagen molecules by the N- and C- proteinases, thereby creating the insoluble collagen molecule [31, 83]. Collagen type I was not found in the absence of MMC at 10 % strain, which contradicts previous studies where mechanical stimulation led to increased collagen I synthesis in TCs [74, 84-86] and BMSCs [28, 46, 87], a process mediated by TGF-β1 [88]. Collagen types III, V and VI were deposited by all cell types, whilst collagen type IV was deposited mostly by BMSCs. Increased deposition of collagen types III, IV, V and VI has been observed in fibroblasts [31] and increased deposition of collagen III has been described in BMSCs and corneal fibroblasts [32], when using carrageenan as a crowder. Further, deposition of collagen type IV by BMSCs has also been increased by a Ficoll™ 70 and 400 kDa cocktail, though this was performed in adipogenic differentiation conditions [89]. Collagen types I and III are typically found together in skin and tendon, as they form heterotypic fibrils, with collagen III localising at the periphery of the fibrils, indicating that it plays and
key role in regulation of fibril growth and diameter during assembly of intermediate fibrils throughout tendon development [90, 91]. While collagen type III is still present in neonatal and adult tissue, its content decreases and it mostly localises to the endotendinum [92], though it is also increased during early healing events [93]. Previous studies indicate that the post-translational events that lead to insoluble collagen type III occur earlier than the ones associated with type I, which correlates with the higher presence of type III at day 3 [94]. However, mechanical stimulation is known to stimulate collagen type III synthesis in TCs [30] and BMSCs [95], which was not seen here. Similarly to collagen type III, collagen type V forms heterotypic fibrils with type I, although it is present in lower amounts, and is necessary for nucleation of type I protofibrils and regulation of lateral growth during tendon development [96-99]. Further, the pericellular localisation of type V procollagen has been suggested to contribute to easier access to proteases, which accelerates its processing to the insoluble form, explaining why MMC did not affect collagen V deposition consistently. Collagen type IV is present in basement membranes and is secreted by epithelial and endothelial cells [100, 101]. Although it is not present in tendon tissue, it can be found at the surface of adult tendon tissue as a single layer of epithelium and is thought to protect from adhesion formation to surrounding tissues [102]. Collagen type IV is secreted by BMSCs and increased deposition has been associated with adipogenic differentiation [103]. Due to the protective and structural role of basement membranes, collagen type IV also has a role in wound healing and is believed to play an important role during tendon injury [102, 104]. Collagen VI is also present in tendon and dermis at different developmental stages [105-107] and it helps modulating type I collagen fibril diameter during fibrillogenesis in tendon, which ultimately affects its tensile strength and stiffness [108, 109].

Gene expression analysis using a TaqMan® array was performed to further assess suitability of the different cell sources for tendon tissue engineering, under the specific in vitro microenvironment modulators utilised herein. This preliminary analysis included commonly accepted tenogenic markers and negative markers to account for the possibility of trans-differentiation towards chondrogenic and osteogenic lineages. Collagen type I is the predominant constituent of tendon ECM, whilst TNC and THBS4, both glycoproteins found in the tendon ECM, play important roles in tendon development and regulation of ECM structure and fibril
organisation during remodelling [110, 111]. Likewise, SCXA, is associated with early stages of tendon development, whereas TNMD, a transmembrane glycoprotein, is present in developing and mature tendon [112, 113]. As for chondrogenic markers collagen types II and X, ACAN and COMP were used, as they are components of cartilage ECM [114]. However, ACAN and COMP are also found in tendon, with ACAN being associated with the tensional region of tendon and fibrocartilage / tendinopathy [115-117] and COMP, member of the thrombospondin family, has a catalyst role in collagen I fibrillogenesis and has been found in tendon regions exposed to compressive load [118-120]. Osteogenic markers included ALPP and IBSP, both indicators of tissue mineralisation [121-125], RUNX2, a transcription factor present in developing bone [126], and SPARC, or osteonectin, a calcium-binding glycoprotein [127]. SPARC is also present in other tissues besides bone, particularly during embryonic development and tissue remodelling [128]. It has been associated with tendon development and it is involved in early stages of collagen fibrillogenesis [129, 130]. Gene expression analysis of TCs was unchanged at day 3, while at day 7 upregulation of SCXA, THBS4 and COMP was observed under mechanical stimulation and absence of MMC. The combination of 10 % strain and MMC upregulated SCXA. These results indicate that the use of 10 % strain can be conducive to tenogenic phenotype maintenance, since SCXA, THBS4 and COMP are expressed in tendon tissue. Contrary to our results, mechanical stimulation is commonly associated with increased collagen type I synthesis in TCs [22, 74]. Further, expression of collagen type III, TNC, TNMD and SCXA is also typically increased [22, 30, 131]. Gene expression of NDFs revealed upregulation of SCXA and THBS4 at day 3 under mechanical stimulation, whilst ADFs presented upregulation of TNC at day 3 and SCXA, THBS4 and COMP at day 7. Although the upregulation of these markers can be indicative of tenogenic trans-differentiation, both cell types expressed ALPP, which is closely associated with the osteogenic phenotype. Moreover, the expression of tenogenic and osteogenic markers can indicate a phenotype similar to what is found in the osteotendinous junction of tendons [132-134]. There were differences in terms of specific response of NDFs and ADFs, which is in agreement with previous work that has shown that foetal, neonatal and adult fibroblasts have different regenerative / healing potential [135-138]. Although dermal fibroblasts have been used in preclinical models [139-142] and in clinic [2, 3] for tendon
regeneration, limited in vitro studies address the issue of their specificity and expression of positive or negative markers that dictate their suitability for this clinical target. In terms of mechanical stimulation with the purpose of tenogenic trans-differentiation, only static strain has been applied to dermal fibroblasts [25, 143]. One study described increased expression of SCXA, TNMD, collagen types I and VI and TNC in aligned electro-spun gelatin fibres for 3 and 7 days under static loading [143]. However, the underlying anisotropic fibre alignment was determinant for the expression of tenogenic markers. Nevertheless, it is important to consider that markers for other phenotypes were not assessed. In addition to micro-grooved / nano-fibrous topographies [144, 145], high density culture has also been described to enhance expression of tenogenic markers in dermal fibroblasts [146]. Mechanical stimulation of dermal fibroblasts has been shown to upregulate gene expression of collagen type I [147], which in this work was not observed. Moreover, TNC has also been found to increase under mechanical stimulation, as it was observed with ADFs at day 3. BMSCs exhibited downregulation of COL1A1 at day 3 under mechanical stimulation, which contradicts previous reports of increased expression of COL1A1, TNC and SCXA under 10 % strain [67, 69, 148, 149]. Nonetheless, most of these studies were performed under continuous mechanical stimulation for 48 hours. Further, a previous report highlights that although COL1A1 gene expression was increased after 1 day of stimulation, at 3 days, the expression levels were comparable to the unloaded control [70]. Additionally, other studies have shown minimal variation of COL1A1 gene expression at 7 days, whereas at 14 days, upregulation was detected [46], clearly demonstrating the changes in gene expression occur at specific time points and can be undetected, if these do not match experimental endpoints. Detection of other tenogenic markers also appeared to be time-sensitive, with TNC only being upregulated after 24 hours of continuous loading, whilst upregulation of collagen types I and III being observed after 12 hours [28]. At day 7, 10 % strain alone induced upregulation of COL10A1 in BMSC cultures. This indicative chondrogenic differentiation (or late stage chondrocyte hypertrophy [150], given the enhanced gene expression of COL10A1) as a function of mechanical loading is not surprising. Previous studies have shown enhanced chondrogenic differentiation of BMSCs under cyclic mechanical stimulation, although it is usually performed under compression [151-153]. This chondrogenic tendency should have been
increased under macromolecular crowding, considering that carrageenan has been shown to favour chondrogenic differentiation in BMSC cultures, maybe due to its sulphated nature. Indeed, numerous studies have demonstrated that sulphated polysaccharides (e.g., chondroitin sulphate, heparan sulphate, dermatan sulphate, dextran sulphate) either as scaffolds [154, 155] or as media supplements [156], maintain chondrogenic phenotype of chondrocytes [157-159] or significantly enhance chondrogenesis in stem cell cultures [160-162]. However, this was not observed at day 7: 10% strain and macromolecular crowding did not later the gene expression profile of BMSCs. We speculate that the enhanced collagen type I deposition, due to macromolecular crowding, may be responsible for this. In any case, further studies are required to determine the influence of various crowding agents’ chemistries on stem cell differentiation and to assess whether crowding is strictly a biophysical phenomenon.

During the course of these experiments, it was visible that some cell detachment occurred from the silicone well-plate, particularly at later time points due to the formation of a confluent rich in ECM cell sheet, which prohibited analysis at later time points. The use of such flexible silicone systems is commonly paired with a surface coating of collagen type I or fibronectin [45, 163]. However, in this case, this would have affected analysis of ECM composition and could have interfered with results derived from the presence of MMC. Further, it is worth noting that this rather soft substrate may have also played a role in cell attachment/orientation, protein deposition and gene expression profile of different cell sources [164-166]. Indeed, it has been well-described in the literature the influence of substrate rigidity in cell phenotype maintenance/commitment [164, 167]. Additionally, rigidity is known to modulate cell morphology, attachment and migration, parameters that are also heavily influenced by cell density [168-170]. Cell density has also been described to play an important role alongside substrate rigidity. Studies suggest that direct cell-cell contact in confluent cultures can alter cell behaviour in substrates of varying stiffness [167, 171]. It is also important to consider that mechanical stimulation protocols for tenogenic phenotype maintenance and differentiation vary widely in terms of strain amplitude, frequency, loading time, substrate topography/chemistry. This lack of standardisation leads to contradictory results in the literature and hinders progress in the field as it impairs result interpretation and comparison. Further, the absence of highly specific tenogenic markers and
knowledge about their spatial and temporal expression in tendon development, maturation and injury is a drawback that needs to be urgently addressed.
4.5 Conclusions

The effect of mechanical loading and macromolecular crowding on tenocytes (phenotype maintenance), neonatal and adult dermal fibroblasts (trans-differentiation) and bone marrow stem cells (differentiation) was assessed. Permanently differentiated cells aligned perpendicularly to the applied load at day 3, whilst bone marrow stem cells started aligning perpendicularly to the applied load at day 7. On protein level, all cells deposited collagen types I, III, V and VI; bone marrow stem cells also deposited collagen type IV. On gene level, only tenocytes appeared to maintain their phenotype, considering the observed upregulation of scleraxis. Trans-differentiation or differentiation towards tenogenic lineage were not observed, considering that neonatal dermal fibroblasts upregulated scleraxis and alkaline phosphatase, adult dermal fibroblasts upregulated thrombospondin 4 and cartilage oligomeric protein and no difference was observed in bone marrow stem cells cultures. The data contribute to a better understanding of the influence of biophysical cues on permanently differentiated and stem cell lineage commitment. They further suggest that biophysical cues alone may not be substantial enough to drive differentiation / trans-differentiation.
4.6 References


Chapter 5 - Summary, future directions and conclusions
5.1 Introduction
Current treatments for tendon repair do not allow for full recovery of functionality [1]. Biomaterial and tissue engineering approaches have attempted to recreate the complex tendon microenvironment, but have failed to direct cell phenotypic commitment and to imitate the highly ordered structure of the native tissues [2]. The aim of this study was to recreate different aspects (macromolecular crowding, oxygen tension, mechanical stimulation) of the native tendon milieu \textit{in vitro} to better modulate the phenotype of several cell sources (tenocytes, dermal fibroblasts, bone marrow derived mesenchymal stem cells) for fabrication of an \textit{in vitro} tendon equivalent.

5.2 Summary
Tendon injuries constitute an unmet clinical need, with 3 to 5 million new incidents occurring annually worldwide. Tissue grafting and biomaterial-based approaches fail to provide environments that are conducive to regeneration; instead they lead to nonspecific cell adhesion and scar tissue formation, which collectively impair functionality. Cell based therapies may potentially recover native tendon function, if tenocyte trans-differentiation can be evaded and cell differentiation / trans-differentiation towards tenogenic lineage is attained. To this end, recreating an artificial \textit{in vivo} tendon niche by engineering functional \textit{in vitro} microenvironments is a research priority. In this work, the effect of biophysical (macromolecular crowding, mechanical stimulation) and biochemical (oxygen tension) modulators on the behaviour of permanently differentiated cell sources (human adult and neonatal dermal fibroblasts and tenocytes) and stem cells (bone marrow mesenchymal stem cells) was assessed.

Firstly, the influence of hydrodynamic radius, charge and polydispersity of various concentrations of different crowders and various cocktails on the extracellular matrix deposition in human dermal fibroblast culture was analysed. Carrageenan was found to induce the highest extracellular matrix deposition due to its natural polydispersity and negative charge, conducive to more efficient volume exclusion. Cocktails of different molecular weight / concentrations of Ficoll™ or dextran sulphate, although presented increased polydispersity than their mono-domain counterparts, did not match carrageenan’s performance.
Combining the optimal macromolecular crowding conditions with low oxygen tension, maintained the multi-differentiation potential of bone marrow mesenchymal stem cells, though adipogenesis was reduced after pre-treatment with 2 % oxygen tension and chondrogenesis increased after pre-conditioning with macromolecular crowding.

Finally, the combined effect of macromolecular crowding and mechanical stimulation allowed for accelerated collagen deposition and fabrication of highly aligned tenocytes and dermal fibroblasts sheets, with gene expression data indicating suitability of this multifactorial approach for tenogenic phenotype maintenance.

Taken together, these results provide further knowledge on the use of biophysical and biochemical in vitro microenvironment modulators for control of cell phenotype and development of tissue-like assemblies, paving the way for advancements in cell therapies for tendon repair and regeneration.

5.3 Limitations
Throughout the course of this work, several limitations arose. In terms of the characterisation and optimisation of MMC conditions, one major limitation was the use of dynamic light scattering (DLS). It did not allow for determination of hydrodynamic radius and polydispersity of lower concentration solutions of dextran sulphate and it would have been more suitable to resort to techniques such as nanoparticle tracking analysis. Considering that bone marrow stem cells under hypoxia did not lead to a further increase in collagen synthesis, other oxygen tensions and an in-depth analysis of the hypoxia-inducible factors and their signalling pathways could have helped to better understand the mechanism by which oxygen tension affects matrix synthesis. Regarding the tenogenic phenotype maintenance and differentiation using mechanical loading and macromolecular crowding, one major limitation is the lack of well-defined markers for tenogenic lineage. Although in this work, this obstacle was indirectly tackled by assessing negative markers (osteogenic and chondrogenic lineages), the widely accepted positive markers (e.g. collagen types I and III, tenomodulin, scleraxis, tenascin-C), which were also assessed herein, are also shared by other cell types. Moreover, the nature of bioreactor systems currently available is an inherent limitation to any study involving mechanical stimulation. The silicone substrates typically used in
scaffold-free set-ups for mechanical loading led to cell detachment that hindered the continuation of the study past day 7. Further, dynamic loading systems are expensive and allow for running of a limited sample number at each time, which hinders study design and analysis, as well as upscaling prospects. Finally, a common limitation to all chapters is the fact that the tissue created in vitro has not been assessed in vivo and, therefore, the clinical benefit of the use of these microenvironment modulators to fabricate clinically relevant implants is yet to be demonstrated.

5.4 Future directions
Considering the limitations and other questions that have surfaced throughout the course of this work, some research areas of further interest are outlined below.

5.4.1 In vivo assessment of tenocyte, dermal fibroblast and bone marrow stem cell sheets for tendon repair
Considering the potential for tenogenic phenotype maintenance, transdifferentiation and differentiation demonstrated by mechanical stimulation and macromolecular crowding in vitro, further pre-clinical testing (window or full defect) should be carried out to assess the suitability of the different cell sources in tendon repair and regeneration. Cell sheets of different cell types (e.g. tenocytes, dermal fibroblasts, bone marrow stem cells) can be fabricated in vitro in the presence of macromolecular crowders to accelerate extracellular matrix deposition [3]. To fabricate scaffold-free implants, cell sheets would be detached and rolled (the number of layers has to be optimised as cell survival can be compromised in the middle layers [4]) as has been described previously [5, 6] to create a stronger construct that could sustain further maturation under appropriate mechanical stimulation (static or dynamic). In vitro maturation of scaffold-free constructs has been described previously in the literature for the fabrication of blood vessels [7], though it required a prolonged culture period. It is anticipated that macromolecular crowding would significantly reduce the time required to develop an implantable device (time to be determined). If the produced constructs are still of not sufficient mechanical integrity, then a bidirectionally aligned scaffold (e.g. electro-spun mats) would have to be used.
5.4.2 Influence of mechanical stimulation, topographical cues and macromolecular crowding on the lineage commitment of bone marrow stem cells

Mechanical stimulation is widely used to induce stem cell differentiation towards tenogenic, osteogenic and chondrogenic lineages [8-10]. However, mechanical loading regimens described in the literature vary widely and results regarding expression of specific phenotypic markers are not comparable or conclusive. Moreover, the use of topographical cues appears to be determinant for the phenotypic commitment even when combined with mechanical stimulation [11] or growth factor supplementation [12]. The characteristics of the mechanical stimulation regime applied can be determinant in directing stem cell phenotypic commitment [13]. Further, the use of such biophysical cues is known to affect the composition of the secreted extracellular matrix [14] and deposition of this tissue-specific extracellular matrix can be accelerated by macromolecular crowding. Therefore, the use of mechanical stimulation (different strain amplitudes, loading times and insertion of rest periods) and topography (e.g. aligned grooves) can direct the cell phenotype towards different lineages (e.g. tendon, cartilage, bone) and consequently modulate extracellular matrix secretion, whilst macromolecular crowding (e.g. carrageenan, Ficoll™, dextran sulphate) can accelerate the deposition of tissue-specific extracellular matrix.

5.4.3 Identification of tendon-specific markers through genomic and proteomic characterisation of tendon tissue from different species

One of the major limitations of the tendon tissue engineering and regeneration field is the lack of specific markers that identify tenocytes [15]. Currently accepted tenogenic markers are shared by various other cell sources and, although osteogenic and chondrogenic markers can be used as negative controls, some of these are also expressed in tendon at different levels and stages [16, 17]. Therefore, adult tendon tissue from different species (human, large and small animals) should be characterised in terms of gene and protein expression using high throughput screening techniques (e.g. microarrays, mass spectrometry), compared with adult cartilage and bone to establish unique markers and relative levels of expression and further analysed through Ingenuity® Pathway Analysis. Additionally, relevant
markers can then be assessed in tenocyte culture in vitro to analyse phenotypic drift [18].

5.5 Conclusions
Macromolecular crowding was used to modulate the in vitro culture of human dermal fibroblasts and the effect of different physical parameters on extracellular matrix deposition was assessed. Carrageenan was shown to be the most efficient crowder in terms of volume exclusion and consequent accelerated matrix deposition due to its high natural polydispersity and negative charge. Macromolecular crowding was then combined with other biochemical (hypoxia) and biophysical (mechanical stimulation) cues to further enhance extracellular matrix synthesis and modulate cell phenotype. However, it was observed that neither low oxygen tension (in bone marrow stem cells) or mechanical stimulation (in human tenocyte, dermal fibroblast and bone marrow stem cell culture) increased extracellular matrix synthesis. More specifically, pre-conditioning with hypoxia reduced adipogenic differentiation potential of bone marrow stem cells, while pre-treatment with crowding enhanced chondrogenesis. Further, mechanical stimulation and macromolecular crowding were shown to be suitable for fabrication of aligned rich in extracellular matrix cell sheets using tenocytes, neonatal and adult dermal fibroblasts. Although tenocytes appeared to maintain their phenotype, trans-differentiation and differentiation of both adult and neonatal dermal fibroblasts and bone marrow stem cells, respectively, was not achieved since they did not present upregulation of tenogenic markers.

Overall, this work contributed for further understanding of the modulation of the excluded volume effect in vitro and its role in biological reactions alongside its synergistic effect with hypoxia and mechanical stimulation. Moreover, it paves the way for development of cell based therapies for tendon regeneration based on multifactorial approaches that use biochemical and biophysical cues.
5.5 References


## A List of reagents

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<td>Biocolor, United Kingdom</td>
</tr>
<tr>
<td>Skim milk powder</td>
<td>Sigma Aldrich, Ireland</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td></td>
</tr>
<tr>
<td>Sodium dodecyl sulphate 20% solution</td>
<td>Sigma Aldrich, Ireland</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td></td>
</tr>
<tr>
<td>SuperSignal™ West Pico chemiluminescent substrate</td>
<td>Thermo Scientific, Ireland</td>
</tr>
<tr>
<td>Transcriptor First Strand cDNA synthesis kit</td>
<td>Roche, Ireland</td>
</tr>
<tr>
<td>Transforming growth factor beta 3</td>
<td>Lonza, United, Kingdom</td>
</tr>
<tr>
<td>TRI Reagent ®</td>
<td>Sigma Aldrich, Ireland</td>
</tr>
<tr>
<td>Tris base</td>
<td>Fisher Chemicals, Ireland</td>
</tr>
<tr>
<td>Triton® X-100</td>
<td>Sigma Aldrich, Ireland</td>
</tr>
<tr>
<td>Trypan blue</td>
<td>Life Technologies, Ireland</td>
</tr>
<tr>
<td>Trypsin / EDTA</td>
<td>Sigma Aldrich, Ireland</td>
</tr>
<tr>
<td>Tween-20</td>
<td></td>
</tr>
<tr>
<td>Weigert’s Haematoxylin</td>
<td>Sigma Aldrich, Ireland</td>
</tr>
<tr>
<td>Xylene</td>
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</tr>
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</table>
### Table A.2: List of primary antibodies and respective suppliers

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta actin</td>
<td>Abcam, United Kingdom</td>
</tr>
<tr>
<td>Collagen I</td>
<td></td>
</tr>
<tr>
<td>Collagen III</td>
<td></td>
</tr>
<tr>
<td>Collagen IV</td>
<td></td>
</tr>
<tr>
<td>Collagen V</td>
<td></td>
</tr>
<tr>
<td>Collagen VI</td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Sigma Aldrich, Ireland</td>
</tr>
<tr>
<td>Laminin</td>
<td></td>
</tr>
<tr>
<td>Scleraxis</td>
<td>Abcam, United Kingdom</td>
</tr>
<tr>
<td>Tenomodulin</td>
<td></td>
</tr>
</tbody>
</table>

### Table A.3: List of secondary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor® 488 Goat anti - mouse</td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor® 555 Goat anti - mouse</td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor® 488 Goat anti – rabbit</td>
<td>Life Technologies, Ireland</td>
</tr>
<tr>
<td>HRP conjugated goat anti – mouse</td>
<td></td>
</tr>
<tr>
<td>HRP conjugated goat anti - rabbit</td>
<td></td>
</tr>
</tbody>
</table>
Appendices

B List of protocols

B.1 Cell culture

B.1.1 Culture medium preparation for human dermal fibroblasts and human tenocytes
1. Dulbecco’s Modified Eagle’s Medium (DMEM) high glucose (4500 mg/L)
2. 10% Foetal bovine serum (FBS)
3. 1% Penicillin streptomycin

B.1.2 Culture medium preparation for human bone marrow derived mesenchymal stem cells
1. Alpha – Minimum Essential Medium with GlutaMax™ (α-MEM)
2. 10% FBS
3. 1% Penicillin streptomycin

B.1.3 Medium preparation for macromolecular crowding
1. Growth medium for the required cell type (as described above)
2. L-ascorbic acid 2-phosphate (100 µM)
3. Crowder (carrageenan, dextran sulphate or Ficoll™ as described in detail in each section).

B.1.4 Cell thawing and passaging
1. Remove vial from liquid nitrogen container and thaw in water bath at 37°C.
2. Transfer contents to culture flask of appropriate size and add pre-warmed culture medium.
3. Change medium every 2-3 days and monitor cell proliferation with a phase contrast microscope.
4. When cells cover more than 80% of the culture flask, remove culture medium, wash cell layer with Hank’s Balanced Salt Solution (HBSS) and add 5 ml of trypsin / EDTA. Incubate at 37°C for 5 minutes until cells start detaching.
5. Add 5ml of culture medium to neutralise the action of trypsin / EDTA and transfer flask contents into a tube and centrifuge at 1200 rpm for 5 minutes.
6. Discard the supernatant and ressuspend cells in desired amount of medium.
B.1.5 Cell freezing

1. Aspirate culture medium and wash cell layer with HBSS.
2. Add trypsin / EDTA and incubate at 37ºC for 5 minutes.
3. Add culture medium to neutralise the action of trypsin, collect flask contents into a tube and centrifuge at 1200 rpm for 5 minutes.
4. Resuspend supernatant in 1 ml of medium and count cells using a Neubauer chamber.
5. Resuspend cells in necessary amount of freezing medium (90% growth medium with 10% of DMSO) to have 1 million cells per millilitre of medium.
6. Add 1 ml of cell suspension per cryogenic vial and place in Mr. Frosty overnight at -80 ºC.
7. Move to liquid nitrogen for long term storage.
B.2 alamarBlue® assay

1. Prepare a 10 % alamarBlue® solution in HBSS.
2. Remove culture medium from the cells and wash with HBSS.
3. If using a scaffold, move scaffold to new well plate.
4. Add 1 ml of the diluted alamarBlue® solution to the cells and a negative control of alamarBlue® at 10 % alone.
5. To obtain the background absorbance, add HBSS to empty wells.
6. Incubate for 3 hours at 37 ºC, 5 % CO₂.
7. Transfer 100 µl of the alamarBlue® solution and of the negative control and background to a clear 96 well plate.
8. Measure the absorbance at 550 nm and at 595 nm.
9. Subtract the values of HBSS to the values of alamarBlue® alone from both absorbances to obtain the absorbance of alamarBlue®. For 550 nm this value is called absorbance of the oxidised form at lower wavelength (AO₅₅₀) and for 595 nm it is called absorbance of the oxidised form at higher wavelength (AO₅₉₅).
10. Calculate the correlation factor:
    \[ R₀ = \frac{AO_{550}}{AO_{595}} \]
11. To calculate the percentage of alamarBlue® reduced (AR) by the cells use the following:
    \[ AR = A_{550} - (A_{595} \cdot R₀) \times 100 \]
B.3 Live / Dead assay

1. Prepare staining solution by diluting calcein AM to 4 µM and ethidium homodimer-1 to 2 µM in HBSS.
2. To prepare a negative control, sample can be immersed in dimethyl sulfoxide (DMSO) to kill all cells before staining.
3. Remove culture medium from the cells and wash cells with HBSS.
4. Add staining solution to cells (enough volume to cover completely the sample).
5. Incubate at 37 ºC, 5 % CO₂ for 30 minutes.
6. Image under inverted fluorescence microscope:
   - Calcein AM: use FITC filter
   - Ethidium homodimer-1: use Texas Red filter
B.4 Cytotox 96® Non-Radioactive Cytotoxicity Assay

1. At each time point, collect culture medium and keep in 1.5 ml Eppendorf tubes and freeze at -20 ºC or -80 ºC- (the use of phenol free medium is recommended).
2. To prepare the standard curve, prepare various cell dilutions, each in a final volume of 100 µl (this will depend on initial cell seeding density).
3. Lyse cells by adding 10 µl of lysis solution per 100 µl of cell suspension.
4. Centrifuge at 250 g for 4 minutes and discard pellet. Transfer 50 µl of the supernatant to a 96 well plate.
5. Reconstitute substrate mix using assay buffer.
6. Add 50 µl of reconstituted substrate mix to each sample.
7. Incubate plate for 30 minutes at room temperature protected from the light.
8. Add 50 µl of stop solution to each well.
9. Record absorbance at 490 nm.
B.5 Cytoskeleton and nuclei staining

1. At each time point remove culture medium and wash cell layer with HBSS.
2. Fix the cell layer with 2% paraformaldehyde (PFA) for 15 minutes at room temperature.
3. Remove PFA and wash briefly with HBSS.
4. Add 0.2% of Triton-X100 (enough to cover the sample) and incubate for 5 minutes at room temperature.
5. Remove Triton-X100 and wash with phosphate buffered saline (PBS).
6. Add FITC-phalloidin diluted in PBS (1:500) and incubate at room temperature for 1 hour.
7. Wash samples briefly with PBS.
8. Incubate with DAPI in 1x PBS for 5 minutes at room temperature.
9. Image under the inverted fluorescence microscope.
B.6 Cell morphometric analysis

1. Open ImageJ software.
2. File > Open > Select Image
3. For merged images: Image > Colour > Split channels
4. Image > Colour > Channels tools > More > select appropriate colour for each channel.
5. Select freehand tool and trace around the nucleus / cytoskeleton of the cell.
6. Analyse > Set measurements > Select Area, Shape descriptors, Feret’s diameter.
7. Analyse > Measure (or Ctr M) to take measurement of selected area.
9. Press Ctr D to mark the outline of measured area.
10. Repeat for each cell.
B.7 Total protein extraction

1. Remove culture medium and wash cell layer with HBSS.
2. Add RIPA buffer with proteinase inhibitor cocktail (approximately 1 ml per 75 cm²) to the cell layer and incubate under agitation at 4 °C for 30 minutes.
3. Scratch plate and collect cell layer
4. Centrifuge samples for 15 minutes at 12000 rpm.
5. Collect supernatant in Eppendorf tubes and freeze at -20°C or -80°C. Discard pellet.
B.8 Total protein quantification using Pierce™ BCA Protein Assay Kit

1. Prepare a 2 mg/ml solution of bovine serum albumin (BSA) and prepare diluted albumin standards as described in Table B.4 below. Dilutions should be prepared in the same diluent as the samples.

2. Prepare working reagent using reagent A and reagent B (50 parts of A:1 part of B). For microplate procedure, use 200 µl of working reagent per sample.

3. Pipette 25 µl of each standard and sample into a microplate well.

4. Add 200 µl of working reagent to each well and mix plate on a shaker for 30 seconds.

5. Cover plate and incubate at 37 ºC for 30 minutes.

6. Cool plate at room temperature and read absorbance at 562 nm on a plate reader.

7. Prepare a standard curve by plotting the absorbance measurement for each BSA standard versus its concentration in µg/ml and determine protein concentration in each sample.

<table>
<thead>
<tr>
<th>Vial</th>
<th>Volume of diluent (µl)</th>
<th>Volume and source of BSA (µl)</th>
<th>Final BSA concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>300 of stock</td>
<td>2000</td>
</tr>
<tr>
<td>B</td>
<td>125</td>
<td>375 of stock</td>
<td>1500</td>
</tr>
<tr>
<td>C</td>
<td>325</td>
<td>325 of stock</td>
<td>1000</td>
</tr>
<tr>
<td>D</td>
<td>175</td>
<td>175 of vial B</td>
<td>750</td>
</tr>
<tr>
<td>E</td>
<td>325</td>
<td>325 of vial C</td>
<td>500</td>
</tr>
<tr>
<td>F</td>
<td>325</td>
<td>325 of vial E</td>
<td>250</td>
</tr>
<tr>
<td>G</td>
<td>325</td>
<td>325 of vial F</td>
<td>125</td>
</tr>
<tr>
<td>H</td>
<td>400</td>
<td>100 of vial G</td>
<td>25</td>
</tr>
<tr>
<td>I</td>
<td>400</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
B.9 Sircol™ soluble collagen assay

4. Label a set of 1.5 ml microcentrifuge tubes. If sufficient test material is available run duplicate samples.

5. Prepare:
   - Reagent blanks – 100 μl of deionised water or 0.5 M acetic acid or fresh cell culture medium or extraction buffer.
   - Collagen standards - use aliquots containing 5, 10 and 50 μg of the collagen reference standard. Make each standard up to 100 μl using the same solvent as the reagent blanks.
   - Test samples - use volumes between 10 and 100 μl and make up to 100 μl. Where there is no previous knowledge of the collagen content 50 or 100 μl of the test material is suggested for a trial run.

6. To each tube add Sircol Dye Reagent (1 ml). (1 ml of dye is required to fully saturate the collagen molecules within a 100 μl sample volume).

7. Cap tubes; mix by inverting contents and place tubes in a gentle mechanical shaker for 30 minutes, (or manually mix at 5 minute intervals). During this time period a collagen-dye complex will form and precipitate out from the soluble unbound dye.

8. Transfer the tubes to a microcentrifuge and spin at 12,000 rpm for 10 minutes. Carefully invert and drain tubes. Important: firmly packing the collagen-dye complex at the bottom of the tubes is required to avoid any pellet loss during draining of unbound dye.

9. Gently layer on 750 μl ice-cold Acid-Salt Wash Reagent to the collagen-dye pellet to remove unbound dye from the surface of the pellet and the inside surface of the microcentrifuge tube.

10. Centrifuge at 12,000 rpm for 10 minutes. Drain the wash into a waste container and carefully remove any fluid from the lip of the tubes using cotton wool buds.

11. Add 250 μl of Alkali Reagent to reagent blanks, standards and samples. Recap tubes and release the collagen bound dye into solution. A vortex mixer is suitable. When all of the bound dye has been dissolved, usually within 5 minutes, the samples are ready for measurement. The colour is light stable, but should be read within 2 to 3 hours. Keep tubes capped until ready to measure absorbance.

12. Transfer 200 μl of each sample to individual wells of a 96 micro well plate, (keep a record map of the contents of each well; A1 to H12). Set the microplate
reader to 555 nm, or the closest matching blue-green colour filter. Measure absorbance against water for the reagent blanks, standards and test samples. Obtain collagen concentrations from the Standard Curve. Duplicates should be close to ± 5 % of their mean value.
Appendices

B.10 Western blotting

B.10.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

10. Tris 1.5M pH 8.8: weigh 45.375 g of Tris-base, add 220 ml of dH2O, adjust pH to 8.8 with concentrated HCl (37 %) and top up to 250ml with dH2O.
11. Tris 1M pH 6.8: weigh 30.25 g of Tris-base, add 220 ml of dH2O, adjust pH to 6.8 with concentrated HCl (37 %) and top up to 250 ml dH2O.
12. 10% SDS: dissolve 5 g in 50 ml dH2O.
13. 10% APS: dissolve 1g in 10 ml dH2O and prepare aliquots of 250 μl and keep at -20˚C.
14. 10 X Running Buffer: weigh 144 g of glycine (192mM), 30.35 g of Tris base (25 mM), 10 g of SDS (0.1 %) and dissolve in 1 L dH2O. Dilute to 1X before using.
15. 4X sample buffer: mix 2.5 ml of Tris 1M pH 6.8, 0.92 g of SDS, 4 ml of glycerol and 1 ml of 1 % bromophenol blue in PBS and top up to 10 ml with dH2O.
16. 1 M DTT stock solution: weigh 1.54 g of dithiothreitol (DTT) and dissolve in 10 ml of sterile dH2O. Prepare 1 ml aliquots and keep at -20 ºC.
17. Gel preparation: Prepare according with tables below depending on molecular weight of protein of interest.

Tabe B.5: Composition of running gel with varying percentages of acrylamide.

Volumes detailed correspond to preparation of two 1 mm gels.

<table>
<thead>
<tr>
<th>Acrylamide %</th>
<th>6%</th>
<th>7%</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH2O (μl)</td>
<td>5300</td>
<td>5000</td>
<td>4600</td>
<td>4000</td>
<td>3300</td>
<td>2300</td>
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<tr>
<td>30% bis acrylamide</td>
<td>2000</td>
<td>2300</td>
<td>2700</td>
<td>3300</td>
<td>4000</td>
<td>5000</td>
</tr>
<tr>
<td>1.5 M Tris pH 8.8 (μl)</td>
<td>2500</td>
<td>2500</td>
<td>2500</td>
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<tr>
<td>10% SDS (μl)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10% APS (μl)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>TEMED (μl)</td>
<td>8</td>
<td>8</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>
Table B.6: Composition of stacking gel. Volumes detailed correspond to the preparation of two 1 mm gels.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O (μl)</td>
<td>2800</td>
</tr>
<tr>
<td>30% bis acrylamide (μl)</td>
<td>660</td>
</tr>
<tr>
<td>Tris 1.0 M pH 6.8 (μl)</td>
<td>500</td>
</tr>
<tr>
<td>10% SDS (μl)</td>
<td>40</td>
</tr>
<tr>
<td>10% APS (μl)</td>
<td>40</td>
</tr>
<tr>
<td>TEMED (μl)</td>
<td>4</td>
</tr>
</tbody>
</table>

Table B.7: Percentage of acrylamide in running gel to be used depending on molecular weight (Mw) of protein on interest.

<table>
<thead>
<tr>
<th>Protein Mw range (kDa)</th>
<th>Percentage of acrylamide in running gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>~5-50</td>
<td>18 %</td>
</tr>
<tr>
<td>~5-60</td>
<td>16 %</td>
</tr>
<tr>
<td>~10-80</td>
<td>14 %</td>
</tr>
<tr>
<td>~20-150</td>
<td>12 %</td>
</tr>
<tr>
<td>~30-200</td>
<td>10 %</td>
</tr>
<tr>
<td>~40-250</td>
<td>8 %</td>
</tr>
<tr>
<td>~60-300</td>
<td>6 %</td>
</tr>
<tr>
<td>~100-400</td>
<td>4 %</td>
</tr>
</tbody>
</table>

18. To prepare the samples, load 5 to 30 μg of protein per lane (sample total protein content should be calculate using the Pierce™ BCA Protein Assay Kit). Prepare 4 x sample buffer with DTT (200 μl of DTT in 1 ml of buffer). Mix 4x sample buffer with DTT with appropriate amount of sample and vortex.

19. Heat samples at 95°C for 5 minutes and centrifuge samples briefly.

20. Load samples in the wells and run gel at 50 V until samples reach the end of the stacking gel, then increase the voltage to 120 V.

B.10.2 Semi-dry transfer

21. Prepare a stock solution of 10x of Tris / Glycine (weigh 30.35 g of Tris-base and 144 g of glycine and dissolve in 1 L of dH₂O).
Appendices

22. Prepare transfer buffer using 10 ml of 10x Tris / glycine, 20 ml of methanol and 70 ml of dH2O.
24. Remove a cassette from the Trans®-Blot Turbo™ System. Place one soaked filter paper pad in the cassette, followed by the membrane, gel and second filter paper pad. Close the lid of the cassette and insert back in the Trans®-Blot Turbo™ System. Run at 25 V, 1 A for 30 minutes.
25. Successful transfer can be confirmed by Ponceau S staining for total protein detection.
26. Ponceau S stain 1% (v/v) can be prepared by diluting 0.033 g of Ponceau S in 10 ml of dH2O and 0.3 ml of glacial acetic acid and topping up to 30 ml with dH2O.
27. Immerse membrane in Ponceau S for 5 minutes.
28. Immerse membrane in a 5 % aqueous solution of acetic acid for 5 minutes, twice and wash the membrane twice with water.
29. Prepare a 10x TBS solution: dissolve 121 g of Tris-base and 40 g on NaCl in 900 ml of dH2O, adjust pH to 7.6 and add dH2O to 1 L.
30. Prepare TBS-T solution by diluting 10x TBS in dH2O and adding 0.1 % of Tween 20.
31. Block the membrane with 5 % low fat milk in TBS-T for one hour at room temperature.
32. Prepare primary antibody solution in 5 % low fat milk in TBS-T and incubate membrane overnight at 4ºC.
33. Wash membrane 3 times for 10 minutes each with TBS-T.
34. Prepare secondary antibody solution in 5 % low fat milk in TBS-T and incubate membrane for 1 hour at room temperature.

B.10.3 Protein detection
35. For peroxidase-conjugated antibodies use Super Signal™ West Pico Chemiluminescent Substrate. Mix reagents in equal parts in an Eppendorf and pour over the membrane. Incubate for 4 minutes protected from light.
36. Place the membranes in a cassette.
37. Visualise results using a photographic film in a dark room. Expose the film to the membrane (exposure time will vary depend on amount of protein of interest /
antibodies) and develop for a few minutes until bands appear. Wash with water for a few seconds and place film in fixative. Wash again and let film air dry.

38. Repeat exposure as needed for optimal detection.

39. Membrane can be reprobed for a new protein of interest.

40. Prepare mild stripping buffer: weigh 15 g of glycine, 1 g of SDS, add 10 ml of Tween 20, adjust pH to 2.2 and bring volume up to 1 L with dH₂O.

41. Wash the membrane twice in stripping buffer for 7 minutes.

42. Wash membrane twice in PBS for 10 minutes.

43. Repeat blocking step and continue with antibody incubation.

**Table B.8:** Primary antibodies and respective dilutions used for western blotting.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scleraxis</td>
<td>Rabbit polyclonal</td>
<td>1:250</td>
</tr>
<tr>
<td>Tenomodulin</td>
<td>Rabbit polyclonal</td>
<td>1:500</td>
</tr>
<tr>
<td>β-actin</td>
<td>Mouse monoclonal</td>
<td>1:2500</td>
</tr>
</tbody>
</table>

**Table B.9:** Secondary antibodies and respective dilutions used for western blotting.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP conjugated</td>
<td>Goat anti mouse</td>
<td>1:5000</td>
</tr>
<tr>
<td>HRP conjugated</td>
<td>Goat anti rabbit</td>
<td>1:5000</td>
</tr>
</tbody>
</table>
B.11 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

B.11.1 Gel / material preparation
1. 1.875 M Tris-HCl, pH 8.8. Dissolve 22.70 g Tris-base (Bio-Rad, 161-0716) in 80 ml ddH₂O; add 2 ml concentrated HCl (37 %), leave it overnight to equilibrate, adjust pH to 8.8 with a few drops concentrated HCl, make it up to 100 ml with ddH₂O. Keep it at 4-8ºC
2. 1.25 M Tris-HCl, pH 6.8. Dissolve 15.14 g Tris-base in 70 ml ddH₂O; add 7 ml concentrated HCl (37 %), leave it overnight to equilibrate, adjust pH to 6.8 with a few drops concentrated HCl, make it up to 100 ml with ddH₂O. Keep it at 4-8ºC
3. 5x sample buffer. Dissolve completely 0.25 g SDS (Bio-Rad 161-0301) in 0.625 ml 1.25M Tris-HCl, pH 6.8 and 2 ml ultrapure water. Leave it overnight for the foam to settle. Top up with glycerol (Bio-Rad) to 5 ml (approximately 2.3 ml). Add 2.5 mg bromophenol blue (Bio-Rad 161-0404) per 10 ml buffer.
4. 5x running buffer. Dissolve 15.1 g Tris-base (Bio-Rad 161-0716), 72 g glycine (Bio-Rad 161-0718) and 5 g SDS (Bio-Rad 161-0301) in 1 L ddH₂O. Store at 4ºC. 1x running buffer is made to run the gel from 5x running buffer by diluting in ddH₂O. Alternatively, 10x Tris-Glycine-SDS 5 L tube can be purchased. 1x running buffer is made to run the gel from 10x running buffer by diluting in ddH₂O.
5. 30% Acrylamide/Bis (37.5:1)
6. 10% SDS
7. 100 mg/ml Ammonium Persulphate in ddH₂O. Dissolve 500 mg APS in 5 ml ddH₂O, aliquot it in eppendorf tubes and keep it at -20ºC. The solution is active for a few months.
8. TEMED
9. 10 % and 70 % Ethanol in dH₂O
10. Phenol Red Solution: dissolve 10 mg of phenol red in 40 ml of ddH₂O, then dilute to 50 ml.

B.11.2 Sample collection and digestion
1. Aspirate the medium from the cell culture and store in appropriate tubes.
2. Wash the cell layer with HBSS
3. Prepare 1 mg/ml pepsin in 0.5 M acetic acid and add to culture medium (100 µl per 1 ml of medium). For cell layer digestion, dilute pepsin solution 1/10 (v/v) in
HBSS to make up a final concentration of 100 µg/ml and add to each well (150 µl / well for 24 well plates)
4. Vortex briefly and place samples on a rotating shaker at 37 °C for 2 hours with continuous shaking at 200 rpm for 2 hours.
5. Scrape off the cell layer using a pipette tip and transfer to tubes. Cell layer samples from 4 wells can be pooled in a 1.5 ml tube.
6. Add 5 µl of phenol red solution in 100 µl to each tube containing cell layer samples (samples will turn yellow).
7. Add 5 µl of 1 M NaOH in 100 µl of cell layer sample. Repeat this step until the samples turn bright pink. Vortex briefly.
8. To neutralize the medium samples, add 20 µl of 1 M NaOH in 1 ml of medium sample. Repeat this step until the samples turn in to pink. Vortex briefly.
9. Store at 4° C for short-term storage or at -20 ° C for long-term storage.

B.11.3 Sample preparation
1. Take the cell layer samples, medium samples and collagen standard in a fresh 1.5 ml centrifuge tube (24 µl each).
2. Add 24 µl of double distilled water in these samples.
3. Add 12 µl of 5X sample buffers to get 5:1 dilution.
4. Vortex the samples and centrifuge them briefly. Store them at 4° C.
5. Prior to SDS-PAGE, denature the samples and standard by heating at 95° C for 5 minutes.
6. Vortex and then centrifuge the samples briefly.
7. Load 15µl per well in Mini gel (for 10-well: 15 µl and for 15-well: 10 µl).
8. Empty well: 15 µl of 1x sample buffer (diluted from 5x sample buffer with water)

Table B.10: Detailed sample preparation for SDS-PAGE.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total volume on the well</th>
<th>Collagen standard / Sample composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mini gel; 10-well or 15-well</td>
<td>For 10-well: 15 µl  For 15-well: 10 µl</td>
<td>24 µl sample  24 µl dH2O  12 µl 5x sample buffer</td>
</tr>
</tbody>
</table>
B.11.4 Gel preparation

1. Clean glass plates with 70% ethanol and wipe dry with tissue paper.
2. Set the gel making apparatus ensuring that the glass plates fit snugly to the platform (mini gel: 1mm space using appropriate spacers).
3. Check for any leaks by pouring water prior to making the gels.
4. Add the gel ingredients to make the 5% resolving gel according to Table B.011.
5. Make sure to add the APS and TEMED last, right before the gels are to be poured.
6. Using a Pasteur pipette, pour the prepared mixture carefully into the space between the 2 glass plates to reach about 1 cm (mini gel) from the bottom of the wells etched out by the comb (keep the excess solution to check how quickly the gels will be polymerised).
7. Overlay the gel with 10% Ethanol to cut off oxygen in contact with the gels.
8. Leave it aside for approximately 30 minutes until set (check with the excess solution remained).
9. During the setting period, prepare the 3% stacking gel according to the Table B.012 (do not add the APS and TEMED until the gel is ready for pouring).
10. A line at the ethanol-gel interface that initially had disappeared will reappear when polymerization is complete.
11. Carefully aspirate the ethanol out of the glass plates using a syringe and imbibe any traces using filter paper.
12. Now add the APS and TEMED to the stacking gel and carefully pour it on top of the polymerised resolving gel. Immediately insert the comb taking care to avoid trapping any air bubbles (keep the excess solution to check how quickly the gels will be polymerised).
13. Allow it to set for 10-15 minutes and, in the meantime, denature samples and standards at 95 °C as described above.
14. After the gels have been set (10 - 15 minutes, check it with the excess solution), remove the combs slowly.
15. Assemble the electrophoresis apparatus, for small gel apparatus, fit the gel plates on the electrode bar and fit the set into the inner chamber and clamp them.
16. Fill the upper/inner chamber with 1x running buffer.
17. Wash the wells by squirting buffer into the wells with a hypodermal needle syringe to remove all air bubbles.
18. Load the standards, samples and markers using Hamilton syringe. Wash the syringe in between using the running buffer in the chamber (at least 5-times).

19. Put the upper chamber on the main chamber, close the lid and run the gel(s)

20. For the mini gel: run at constant voltage (50 V) until the front reaches the end of the stacking gel (± 30 - 40 min), then increase voltage to 120 V until the front reaches the end of the separating gel (± 1 hour).

21. Remove the glass using the wonder wedge, cut the lower right hand corner and release the gel slowly into dH2O.

Table B.011: 5% Separation gel (1 mm thickness) for collagen for mini gel
(Protean II Bio-Rad).

<table>
<thead>
<tr>
<th></th>
<th>1 Gel (µl)</th>
<th>2 Gels (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 % Acrylamide/Bis (37.5:1)</td>
<td>830</td>
<td>1660</td>
</tr>
<tr>
<td>1.875 M Tris-HCl pH 8.8</td>
<td>1000</td>
<td>2000</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>ddH2O</td>
<td>3070</td>
<td>6140</td>
</tr>
<tr>
<td>APS (100 mg/ml)</td>
<td>42</td>
<td>84</td>
</tr>
<tr>
<td>TEMED</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>5000</strong></td>
<td><strong>10000</strong></td>
</tr>
</tbody>
</table>

Table B.012: 3% Stacking gel (1 mm thickness) for collagen for mini gel
(Protean II Bio-Rad)

<table>
<thead>
<tr>
<th></th>
<th>1 Gel (µl)</th>
<th>2 Gels (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 % Acrylamide/Bis (37.5:1)</td>
<td>200</td>
<td>400</td>
</tr>
<tr>
<td>1.25 M Tris-HCl pH 6.8</td>
<td>200</td>
<td>400</td>
</tr>
<tr>
<td>10% SDS</td>
<td>33</td>
<td>66</td>
</tr>
<tr>
<td>ddH2O</td>
<td>1550</td>
<td>3100</td>
</tr>
<tr>
<td>APS (100 mg/ml)</td>
<td>17</td>
<td>33</td>
</tr>
<tr>
<td>TEMED</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2000</strong></td>
<td><strong>4000</strong></td>
</tr>
</tbody>
</table>
B.11.5 Silver staining

To stain the gels obtained in the previous steps the SilverQuest™ Silver Staining kit was used. The procedure for the Basic Protocol is detailed in Table B.13.

Table B.13: Detailed procedure for silver staining.

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Total volume</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fix</td>
<td>40 ml Ethanol 10 ml Acetic acid 50 ml Water</td>
<td>100 ml</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Wash</td>
<td>30 ml Ethanol 70 ml Water</td>
<td>100 ml</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Sensitise</td>
<td>30 ml Ethanol 10 ml Sensitiser 60 ml Water</td>
<td>100 ml</td>
<td>10 minutes</td>
</tr>
<tr>
<td>First wash</td>
<td>30 ml Ethanol 70 ml Water</td>
<td>100 ml</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Second wash</td>
<td>100 ml Water</td>
<td>100 ml</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Stain</td>
<td>1 ml Stainer 99 ml Water</td>
<td>100 ml</td>
<td>15 minutes</td>
</tr>
<tr>
<td>Develop</td>
<td>10 ml Developer 1 drop Developer enhancer 90 ml Water</td>
<td>100 ml</td>
<td>5 – 8 minutes</td>
</tr>
<tr>
<td>Stop</td>
<td>10 ml Stopper (add directly to the Developing solution)</td>
<td>10 ml</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Wash</td>
<td>100 ml Water</td>
<td>100 ml</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>
B.12 Immunocytochemistry

10. At the end of culture time points, aspirate the medium and wash cell layer with HBSS.

11. Fix with 2 % PFA (pre-cooled at 4°C) for 15 minutes.

12. To make 2 % PFA (in glass bottle with magnetic stirrer): Weight 0.2 g of PFA and add 10 ml of PBS. Put on a magnetic stirrer with heater. Leave it for around 1 hour (put the cap on, but loosen it). Cool it and keep it at 4°C.

13. Drain away fixative and wash 3x with PBS, 5 minutes each.

14. Block with 3 % (w/v) BSA in 1x PBS for 30 minutes at room temperature (RT). To make 3 % BSA: weight 0.3 g of BSA and add 10 ml of PBS. Put on a magnetic stirrer and leave it for around 1 hour. Store it at 4°C.

15. Incubate with primary antibody in 1x PBS for 90 minutes at room temperature or overnight at 4°C. List of primary antibodies used can be found in Table B.14.

16. Wash 3x with 1x PBS, 5 minutes each.

17. Incubate with secondary antibody in 1x PBS for 30 minutes at room temperature. List of secondary antibodies can be found in Table B.15.

18. Wash 3x with 1x PBS, 5 minutes each.

19. Incubate with DAPI in 1x PBS for 5 minutes at room temperature.

20. Wash 3x with PBS, 5 minutes each.

21. Image samples on Olympus IX-81 inverted fluorescence microscope.

Table B.14: Primary antibody source and dilution.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>Mouse monoclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Collagen III</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Collagen V</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Collagen VI</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Mouse monoclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Laminin</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
</tr>
</tbody>
</table>
Table B.15: Secondary antibody source and dilution.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor® 488</td>
<td>Goat anti – rabbit</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa Fluor® 488</td>
<td>Goat anti - mouse</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa Fluor® 555</td>
<td>Goat anti - mouse</td>
<td>1:500</td>
</tr>
</tbody>
</table>
B.13 Extraction of bone marrow derived mesenchymal stem cells

1. Determine the required number of culture flasks (T175) required to seed cells at a density of $10^5$ cells/cm$^2$. Mononuclear cell count is provided on the label of the tube.

2. Coat the growth surface of the culture flasks with sterile fibronectin solution (10 ng/ml) in PBS and leave for at least 1 hour.

3. Remove fibronectin solution from culture flask and pipette sufficient volume of culture medium to cover the growth surface of the flask to a depth of 3 to 5 mm.

4. Add the required volume of bone marrow aspirate to each flask and rock gently to disperse evenly.

5. Transfer the culture flasks to an incubator and leave for 7 days.

6. After 7 days, replace 50% of culture medium.

7. Return to the incubator for another 7 days, after which 100% of the medium can be changed. If after removing medium it is still not possible to view the bottom of the flask to assess cell attachment, gently wash with PBS.

8. Marrow stromal cells (P0) that have attached to the flask should be visible under a microscope as colonies of elongated fibroblastic cells.
B.14 Trilineage differentiation of bone marrow derived mesenchymal stem cells

B.14.1 Adipogenic differentiation and Oil Red O staining
1. Seed cells at $2.1 \times 10^4$ cells/cm$^2$ and incubate at 37 °C in a humidified atmosphere of 5 % CO$_2$.
2. Feed the cells every 2 or 3 days by completely replacing the medium until cells reach confluency (5-13 days). Cells must be confluent or post-confluent for optimal adipogenic differentiation.
3. At 100 % confluency, 3 cycles of induction / maintenance will stimulate optimal adipogenic differentiation. Each cycle consists of feeding cells with supplemented adipogenesis induction medium and culture for 3 days, followed by 1 to 3 days of culture in supplemented adipogenic maintenance medium. Feed non-induced cells with only supplemented adipogenic maintenance medium. Adipogenic cells are delicate and care should be used to avoid disrupting lipid vacuoles.
4. After 3 complete cycles of induction / maintenance, culture cells for 7 more days in supplemented adipogenic maintenance medium, replacing medium every 2-3 days.
5. Remove culture medium and briefly wash cells with HBSS.
6. Fix cells in 2 % PFA.
7. Stain with freshly prepared Oil Red O solution for 15 minutes.
8. Rinse with distilled water.
9. Lightly stain nuclei with haematoxylin for 1 minute.
10. Rinse with distilled water.

B.14.2 Osteogenic differentiation and Alizarin Red staining
1. Seed cells at $3.1 \times 10^3$ cells/cm$^2$. Cells may peel during differentiation (wells can be coated with collagen to prevent this).
2. Allow cells to adhere to the wells for 4 to 24 hours and induce osteogenesis by replacing medium with osteogenesis induction medium.
3. Feed cells every 3-4 days for 2-3 weeks with fresh osteogenesis induction medium. Feed non-induced cells with standard growth medium.
4. Osteogenic-induced cells will show changes in cell morphology, from spindle shaped to cuboidal shaped, as they differentiate and mineralise. Gaps may form in...
the post-confluent cell layer and cells may begin to delaminate. If this happens, proceed to analysis, as this indicates calcium deposition.

5. For calcium deposition assays, harvest the cells in calcium free PBS and scrape the cells in the presence of 0.5M HCl.
6. Remove culture medium and wash cells with PBS.
7. Fix with 2 % PFA.
8. Stain with Alizarin red solution for 30 seconds to 5 minutes and observe the reaction microscopically.
9. Shake off excess dye and blot samples.

**B.14.3 Chondrogenic differentiation and safranin O / fast green staining**

1. Prepare aliquots of TGF-β3 by ressuspending lyophilised TGF-β3 in sterile 4 mM HCl supplemented with 1 mg/ml of BSA or human serum albumin (HSA) to a concentration of 20 μg/ml. Each 1 μl of solution will convert 2 ml of incomplete chondrogenic medium into complete medium.
2. Aliquot small volumes of TGF-β3 and store at less than -70 ºC for no more than 6 months.
3. After thawing, aliquot should be centrifuged at low speed to pull the small volume to the bottom of the tube. Add the necessary volume to incomplete medium to prepare fresh complete chondrogenic induction medium. Complete medium must be prepared fresh and used within 12 hours.
4. Calculate the number of pellets required (2.5x10^5 cells are required per pellet).
5. Wash the cells with incomplete chondrogenic medium and centrifuge at 150 g for 5 minutes at room temperature and discard the supernatant.
6. Ressuspend cells in 1 ml of incomplete medium per 7.5x10^5 cells. Centrifuge again and discard medium.
7. Ressuspend cells in complete medium to a concentration of 5x10^5 cells/ml.
8. Aliquot 0.5 ml (2.5x10^5 cells) of the suspension into 15 ml propylene tubes (to prevent attachment to the tube; do not use polystyrene tubes). Centrifuge cells ate 150 g for 5 minutes at room temperature. Do not aspirate the supernatant or ressuspend pellet.
9. Loosen the caps of the tubes one half turn to allow gas exchange and incubate the tubes for 24 hours.
10. Feed the cell pellets every 2-3 days by completely replacing the medium in each tube. Add 0.5 ml of freshly prepared complete chondrogenic medium.

11. After replacing the medium, flick the bottom of the tube to ensure the pellets is free-floating. Loosen the caps and return to incubator.

12. Pellets can be harvested after 14 - 28 days in culture. Pellets can be fixed in formalin or embedded in paraffin for histological processing or prepared for frozen sectioning.

13. Remove culture medium and wash pellets with PBS.

14. Fix and section pellets.

15. Stain with Weigert’s iron haematoxylin solution for 10 minutes.

16. Wash in running tap water for 10 minutes.

17. Stain with fast green solution for 5 minutes.

18. Rinse quickly with 1 % acetic acid solution for no more than 10-15 seconds.

19. Stain with 0.1 % safranin O solution for 5 minutes.

20. Dehydrate and clear with 95 % ethyl alcohol, absolute ethyl alcohol and xylene using 2 changes each for 2 minutes each.
B.15 Total RNA isolation using High Pure RNA isolation kit

1. At each time point remove media from cells.
2. Add 1 ml of TRI Reagent® for each 10 cm² of culture dish area (not dependent on cell number).
3. Use pipette to help lyse cells in culture dish.
4. Incubate at room temperature for 5 minutes.
5. Remove all lysed cells into Eppendorf tubes.
6. Centrifuge at top speed to remove sediments and transfer supernatant into a clean Eppendorf.
7. Add 200 µl of chloroform (without isoamyl alcohol) for every 1 ml of TRI Reagent® used.
8. Vortex for 15 seconds.
9. Incubate at room temperature for 5 minutes.
10. Centrifuge at 13,000 rpm at 4 ºC for 15 minutes.
11. Transfer aqueous phase (colourless top layer) to a clean tube containing the same volume of 70 % ethanol and mix with pipette.
12. Transfer the entire volume to a filter column.
13. Centrifuge at 8.000 g for 15 seconds and discard flow through.
14. Add 500 µl of Wash 1 (black cap) and centrifuge at 8.000 g for 15 seconds and discard flow through.
15. Add 500 µl of Wash 2 (blue cap) and centrifuge at 8.000 g for 15 seconds and discard flow through.
16. Add 200 µl of Wash 2 (blue cap) and centrifuge at 13.000 g for 2 minutes and discard flow through.
17. Place filter in a new tube and add 50 µl of elution buffer and centrifuge at 8.000 g for 1 minute.
18. Take the 50 µl and put them again in the filter column and centrifuge at 8.000 g for 1 minute.
19. Aliquot 45 µl for cDNA synthesis and 5 µl for RNA quantity and quality testing.
B.16 Assessment of RNA quantity and quality

For RNA quantity:
1. Blank NanoDrop 1000 by using 1 µl of DNase free water.
2. Add 1 µl of sample and measure concentration.
   a. For A260/A280: samples are accepted above 1.8 (ratio of ~2 is considered pure), lower than that it might indicate presence of protein, phenol or other contaminants.
   b. For A260/A230: values higher then A260/A280 indicate pure nuclei acid. Expected values are in the range of 2.0 – 2.2. values lower than this indicate contaminants which absorb at 230 nm.

For RNA quality:
1. Prepare Nano gel matrix by placing 550 µl of the gel matrix in the receptacle of a spin filter. Centrifuge at 1.500 g for 10 minutes. Aliquot 65 µl of gel matrix intro 0.5 ml tubes and store them at 4 °C.
2. Vortex Nano dye concentrate for 10 seconds and spin down.
3. Add 1 µl of Nano dye to 65 µl of gel matrix, vortex and spin tube for 10 minutes at 13.000 g.
4. Take a Nano chip and place it in the priming station.
5. Add 9 µl of gel – dye mix to the well marked G.
6. Set a timer for 30 seconds and close the priming station making sure the plunger of the syringe is at 1 ml position.
7. Press the plunger until it is held down by the clip and start timer.
8. Release the plunger and wait 5 seconds and confirm the plunger has moved back at least to the 0.3 ml mark.
9. Pipette 9 µl of the gel - dye mix in remaining wells marked G.
10. Load 5 µl of Nano marker into the well with the ladder symbol and then on all other 12 wells.
11. Pipette 1 µl of ladder into the well marked with the ladder symbol.
12. Pipette 1 µl of each sample into each of the 12 sample wells.
13. Vortex at 2.400 rpm in IKA vortex mixer for 60 seconds.
14. Decontaminate electrodes of Agilent Bioanalyzer using RNase ZAP and DNase free water.
15. Place the chip in Agilent Bioanalyzer and start run.
16. Pure samples will show 2 peaks corresponding to 18S and 28S ribosomal RNA.
17. Samples with a RNA integrity number (RIN) lower than 8 were not used for further experiments.
B.17 cDNA synthesis using Transcriptor First Strand cDNA synthesis kit

All components are to be thawed and kept on ice throughout protocol.

1. Prepare and label tubes according with samples collected.

2. To prepare the enzyme mix add per sample (total 7 µl per sample):
   a. 4 µl of reverse transcriptase buffer
   b. 0.5 µl of RNase inhibitor
   c. 2 µl of deoxy mix
   d. 0.5 µl of reverse transcriptase

3. In the labelled tubes add to each:
   a. 4 µl of DNase free water
   b. 1 µl of oligoDT primers
   c. 7 µl of enzyme mix
   d. Sample volume corresponding to 1 µg of RNA (this will be concentration dependent)

4. Mix gently with pipette (do not vortex).

5. Place tube in thermal block cycler and run program:
   a. 1 hour at 50 ºC
   b. 5 minutes at 85 ºC for inactivation.

6. Store at -20 ºC.
B.18 RealTime ready Custom Panel

1. Thaw the solutions and briefly spin vials in a microcentrifuge before opening. To compensate for pipetting losses prepare mixes with 10% overdosage (one extra sample for every 10)

<table>
<thead>
<tr>
<th>Table B.16: Volume for 384 PCR reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Volume (µl)</strong></td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Water (vial 2)</td>
</tr>
<tr>
<td>Probes master (vial 1)</td>
</tr>
<tr>
<td>Sample</td>
</tr>
<tr>
<td>Total volume (µl)</td>
</tr>
</tbody>
</table>

2. Prepare a tube with the water and Probes Master as detailed in table below (PCR mix). Mix carefully by pipetting up and down. Do not vortex.
3. Pull the foil from the Custom Multiwell Plate
4. Pipette 9 µl of PCR mix into each well of the plate.
5. Add 1 µl of cDNA template (sample) into each well for a total volume of 10 µl.
6. When using negative controls, add 1 µl of untranscribed RNA to the corresponding wells.
7. Seal the well plate with sealing foil.
8. Centrifuge plate for 2 minutes at 1,500 g in a standard swing bucket centrifuge.
9. Transfer the plate to the LightCycler® 480 Instrument and use the PCR program described below.

Detection format: Hydrolysis probe
Filter combination: Dynamic mode. FAM 483 – 533 or 465 – 510
Table B.17: Program details for PCR reactions using RealTime ready Custom Panel

<table>
<thead>
<tr>
<th>Programs</th>
<th>Cycles</th>
<th>Analysis Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-incubation</td>
<td>1</td>
<td>None</td>
</tr>
<tr>
<td>Amplification</td>
<td>45</td>
<td>Quantification</td>
</tr>
<tr>
<td>Cooling</td>
<td>1</td>
<td>None</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temperature Targets</th>
<th>Target (°C)</th>
<th>Acquisition Mode</th>
<th>Hold (hh:mm:ss)</th>
<th>Ramp Rate (°C/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-incubation</strong></td>
<td>95</td>
<td>None</td>
<td>00:10:00</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>None</td>
<td>00:00:10</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>None</td>
<td>00:00:30</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>Single</td>
<td>00:00:01</td>
<td>4.8</td>
</tr>
<tr>
<td><strong>Cooling</strong></td>
<td>40</td>
<td>None</td>
<td>00:00:30</td>
<td>2.5</td>
</tr>
</tbody>
</table>
C Results

C.1 Bone marrow derived mesenchymal stem cell phenotype characterisation

After extraction from unprocessed bone marrow, BMSCs were characterised by flow cytometry. Cells belonged to a male donor, 23 years old. This was performed using the Stemflow™ Human MSC Analysis Kit as per manufacturer’s protocol (BD Biosciences, UK).³

Flow cytometry was performed by Andreia Ribeiro (Regenerative Medicine Institute (REMEDEI), National University of Ireland Galway).

Figure C.1: Flow cytometry of extracted BMSCs showing positive expression of surface markers CD90, CD44, CD105 and CD73 and absence of negative markers CD45, CD34, CD11b, CD19, HLA-DR, represented as negative cocktail.

³ Flow cytometry was performed by Andreia Ribeiro (Regenerative Medicine Institute (REMEDEI), National University of Ireland Galway).
C.2 Total protein quantification

**Figure C.2:** Total protein quantification of (A) TCs, (B) NDFs, (C) ADFs and (D) BMSCs in the absence (-MMC) and presence (+MMC) of macromolecular crowding under 0 and 10% strain at days 3 and 7.
C.3 Western blotting optimisation

Two antibodies for western blotting were tested (ab203676 and ab81328) for tenomodulin detection, as this is a commonly accepted tenogenic marker. However, both were highly unspecific as seen in Figure C.3. One antibody led to the detection of multiple bands, while the other one detected one band with the wrong molecular weight. Tenomodulin is expected at 37 kDa. Due to these results this technique was not further pursued.

Figure C.3: Western blotting for detection of tenomodulin with different antibodies.
C.4 Assessment of RNA quantity and quality

**Table C.18:** RNA quantification and integrity number (RIN) of ADFs.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Group</th>
<th>Concentration (ng/µl)</th>
<th>A260/A280</th>
<th>RIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 3</td>
<td>0 % -MMC</td>
<td>161.85</td>
<td>2.08</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>0 % +MMC</td>
<td>121.55</td>
<td>2.06</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>10 % -MMC</td>
<td>114.4</td>
<td>2.8</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>10 % +MMC</td>
<td>449.5</td>
<td>2.15</td>
<td>9.9</td>
</tr>
<tr>
<td>Day 7</td>
<td>0 % -MMC</td>
<td>178.63</td>
<td>2.10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>0 % +MMC</td>
<td>147.3</td>
<td>2.11</td>
<td>10</td>
</tr>
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<td></td>
<td>10 % -MMC</td>
<td>84.8</td>
<td>2.09</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>10 % +MMC</td>
<td>94.07</td>
<td>2.10</td>
<td>9.6</td>
</tr>
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</table>

**Table C.19:** RNA quantification and integrity number (RIN) of NDFs.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Group</th>
<th>Concentration (ng/µl)</th>
<th>A260/A280</th>
<th>RIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 3</td>
<td>0 % -MMC</td>
<td>167.1</td>
<td>2.05</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>0 % +MMC</td>
<td>208.35</td>
<td>2.06</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>10 % -MMC</td>
<td>113.4</td>
<td>2.03</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>10 % +MMC</td>
<td>121.9</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Day 7</td>
<td>0 % -MMC</td>
<td>459.7</td>
<td>2.09</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>0 % +MMC</td>
<td>102.3</td>
<td>2.18</td>
<td>9.9</td>
</tr>
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<td>10 % -MMC</td>
<td>322.2</td>
<td>2.01</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>10 % +MMC</td>
<td>238.2</td>
<td>2.08</td>
<td>9.9</td>
</tr>
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</table>

**Table C.20:** RNA quantification and integrity number (RIN) of TCs.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Group</th>
<th>Concentration (ng/µl)</th>
<th>A260/A280</th>
<th>RIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 3</td>
<td>0 % -MMC</td>
<td>173.9</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>0 % +MMC</td>
<td>119</td>
<td>1.91</td>
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<td>10 % -MMC</td>
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<td>10</td>
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<td></td>
<td>10 % +MMC</td>
<td>471.9</td>
<td>2.13</td>
<td>10</td>
</tr>
<tr>
<td>Time point</td>
<td>Group</td>
<td>Concentration (ng/µl)</td>
<td>A260/A280</td>
<td>RIN</td>
</tr>
<tr>
<td>------------</td>
<td>---------</td>
<td>-----------------------</td>
<td>-----------</td>
<td>-----</td>
</tr>
<tr>
<td>Day 3</td>
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<td>380.2</td>
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</tr>
<tr>
<td></td>
<td>0 % +MMC</td>
<td>172.7</td>
<td>1.99</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>10 % -MMC</td>
<td>94.7</td>
<td>2.3</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>10 % +MMC</td>
<td>185.4</td>
<td>2.07</td>
<td>9.8</td>
</tr>
<tr>
<td>Day 7</td>
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<td>355.15</td>
<td>2.12</td>
<td>10</td>
</tr>
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<td>0 % +MMC</td>
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</tr>
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<td>9.4</td>
</tr>
<tr>
<td></td>
<td>10 % +MMC</td>
<td>85.3</td>
<td>2.06</td>
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</tr>
</tbody>
</table>

**Table C.21**: RNA quantification and integrity number (RIN) of BMSCs.
D Research outputs

D.1 Awards
1. 10th World Biomaterials Congress 2016 Trainee Award, 17th-22nd May 2016, Montréal, Canada.
2. Prize for Best Materials Presentation by IOM3 at the UK Society for Biomaterials Annual Conference 2015, 25th-26th June 2015, Belfast, UK.
3. 2nd Prize for a Podium Presentation at the UK Society for Biomaterials Annual Conference 2015, 25th-26th June 2015, Belfast, UK.

D.2 Publications
2. D. Gaspar, K. P. Fuller, D. Zeugolis, Polydispersity is key modulator of extracellular matrix deposition under macromolecular conditions, Submitted.


**D.3 Book chapters**


**D.4 Conference presentations**


12. **D. Gaspar**, D. Cigognini, P. Kumar, A. Satyam, S. Alagesan, C. Sanz-Noguès, M. Griffin, T. O’Brien, A. Pandit, D. Zeugolis, A multifactorial approach towards enhanced ECM deposition and maintenance of hMSCs phenotype using...
macromolecular crowding and low oxygen tension, Poster Presentation, 4th TERMIS World Congress, 8th-11th of September 2015, Boston, USA.


Appendices

**Presentation**, European Society for Biomaterials, 31st of August - 3rd of September 2014, Liverpool, United Kingdom.

