Unravelling the role of mechanical cues on the chondrogenic differentiation of stem cells

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Trinity College Dublin

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Supervisor

Prof. Daniel J. Kelly
Declaration

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____________________
Paola Aprile
Summary

Untreated cartilage injuries typically progress into degenerative joint disease or osteoarthritis (OA) which is the fastest growing cause of disability worldwide. With increased life expectancy and rising levels of obesity across Europe, OA is predicted to become the fourth leading cause of disability worldwide by 2020\(^1\). Such patients are potential candidates for cell-based or tissue engineering-based treatment solutions, such as biological cartilage regeneration/repair and joint resurfacing. Mesenchymal stem cells / stromal cells (MSC) represent a promising cell type for cartilage tissue engineering, due to their relative ease of isolation, ability to proliferate extensively \textit{in vitro} and differentiate along multiple pathways\(^2-4\). However, understanding the optimal environmental conditions to unlock the potential of these cells will be crucial to developing new cartilage tissue engineering strategies suitable for clinical translation.

The fate of MSC is regulated by the mechanical properties of the local extracellular matrix (ECM). This has been demonstrated for MSC seeded onto material surfaces\(^5,6\), i.e. two-dimensional (2D culture, and within three-dimensional (3D)\(^7-9\) hydrogels. The objective of this thesis was to examine the influence of intrinsic and extrinsic mechanical cues on the initiation of chondrogenesis for MSC seeded on top (2D) or encapsulated within (3D) hydrogels of defined stiffness.

Initially, we developed an Interpenetrating Network (IPN) hydrogel system that supported cell growth in 2D and 3D environments. The IPN system enabled the independent control of substrate stiffness (in 2D and in 3D) and cell morphology (3D only). In the first phase of the thesis, MSC were seeded at varying densities on top of (2D culture) IPN hydrogels of different stiffness. The initiation of chondrogenesis was enhanced by a soft substrate and a high cell density. In these
conditions, MSC could migrate and condensate, forming large aggregates expressing N-Cadherin (NCAD) and upregulating marker genes of chondrogenesis.

In the next phase of the thesis, the 3D IPN system was employed to tailor both substrate stiffness and MSC morphology. As expected, encapsulation of MSC within a IPN hydrogel (3D culture) enhanced chondrogenesis compared to a 2D environment, which was further increased by allowing MSC to spread in the soft 3D IPN. Such compliant 3D environment supported MSC motility and increased cellular condensation. Surprisingly, cells with a rounded morphology, embedded within a soft 3D IPN, expressed higher levels the osteogenic markers RUNX2 and COL1.

The next stage of this thesis explored whether the application of a mechanical stimulus integral to the join environment, specifically cyclic hydrostatic pressure (HP), was sufficient to initiate chondrogenesis of MSC. The response of MSC at different stages of (growth factor induced) chondrogenic differentiation to the application of HP was also assessed. It was found that the application of physiological levels of HP alone wasn’t sufficient to initiate chondrogenesis of MSC. Moreover, the application of HP during the early stages of transforming growth factor (TGF)β-3 mediated differentiation was found to suppress the expression of chondrogenic markers and enhance the expression of osteogenic markers. This was not observed when HP was applied during the later stages of MSC differentiation, in particular after MSC were allowed to condensate.

The development of novel tissue engineering strategies will require further examination of how environmental cues determine cell lineage specification in vitro. In particular, a more mechanistic approach to stem cell mechanobiology may ultimately allow us to understand how to design biomaterials to facilitate specific molecular mechanisms of mechanotransduction. The insight provided by this
dissertation suggests that a mechanobiology informed approach to biomaterial development, with tuneable control of cell-cell interactions and condensation, will be integral to the development of successful cartilage tissue engineering strategies.
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Nomenclature

This section contains some of the abbreviations used in this thesis. It is not a full list of all abbreviations and symbols used – these will be explained in the text whenever used.

2D 2-dimensional
3D 3-dimensional
ACAN Aggrecan
ACI Autologous chondrocyte implantation AGC Aggrecan
ALP Alkaline phosphatase
ANOVA Analysis of variance
ATP Adenosine triphosphate
BSA Bovine serum albumin
Ca++ Calcium ion
CaCl₂ Calcium chloride
CDM Chondrogenic differentiation medium
cDNA Complementary DNA
CHP Cyclic hydrostatic pressure
COL1 Collagen type I alpha 1 chain
COL2 Collagen type II alpha 1 chain
COLX Collagen type 10 alpha 1 chain
CO₂ Carbon dioxide
DAPI 4’,6-diamidino-2-phenylindole
DNA Deoxyribonucleic acid
ECM Extracellular matrix
EDTA Ethylenediamine-tetraacetic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>FA</td>
<td>Focal adhesions</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FIJI</td>
<td>Fiji is just ImageJ</td>
</tr>
<tr>
<td>FS</td>
<td>Free-swelling (unloaded)</td>
</tr>
<tr>
<td>GAG</td>
<td>Sulphated glycosaminoglycan</td>
</tr>
<tr>
<td>GM</td>
<td>Growth Medium</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
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<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HDAC4</td>
<td>Histone deacetylases 4</td>
</tr>
<tr>
<td>HP</td>
<td>Intermittent hydrostatic pressure</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1 beta</td>
</tr>
<tr>
<td>IPN</td>
<td>Interpenetrating networks</td>
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<td>Insulin transferrin selenium</td>
</tr>
<tr>
<td>kPa</td>
<td>Kilo pascal</td>
</tr>
<tr>
<td>LPM</td>
<td>Lateral plate mesoderm</td>
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<td>M</td>
<td>Molar</td>
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<tr>
<td>MACI</td>
<td>Matrix-induced autologous chondrocyte implantation mg</td>
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<td>min</td>
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<td>MPa</td>
<td>Mega Pascal</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribosomal nucleic acid</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell / multipotent stromal cell</td>
</tr>
<tr>
<td>n</td>
<td>Number of observations</td>
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<tr>
<td>N</td>
<td>Newton</td>
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<tr>
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<td>N-Cadherin</td>
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<td>Nitrogen</td>
<td>N2</td>
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<td>ng</td>
<td>Nanogram</td>
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<td>Nanomolar</td>
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<td>Nanometre</td>
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<td>nonmuscle myosin II</td>
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<td>O₂</td>
<td>Oxygen</td>
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<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimum cutting temperature medium</td>
</tr>
<tr>
<td>p</td>
<td>Threshold significance level</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>px</td>
<td>Pixel</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-Glycine-Aspartic acid</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RUNX2</td>
<td>Runt-relates transcription factor 2</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SEM</td>
<td>Standard error from the mean</td>
</tr>
<tr>
<td>SM</td>
<td>Starvation medium</td>
</tr>
<tr>
<td>SMAD 2/3</td>
<td>Mothers against decapentaplegic homolog 2 and 3</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>SOX9</td>
<td>SRY-box transcription factor 9</td>
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<td>t</td>
<td>Time</td>
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<tr>
<td>TAZ</td>
<td>WW domain-containing transcription regulator protein 1</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
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<tr>
<td>TGFBR2</td>
<td>Transforming growth factor-beta receptor 2</td>
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<td>U</td>
<td>Units</td>
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<tr>
<td>UV</td>
<td>Ultraviolet light</td>
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<td>Wet weight</td>
</tr>
<tr>
<td>YAP</td>
<td>Yes associated protein</td>
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Presentations resulting from this study


Aprile P., Sathy B.N., Carroll S.F., Kelly D.J., 2017. *Engineering hydrogel environments to promote chondrogenesis and myogenesis of mesenchymal stem cells.* Proceedings of Bioengineering in Ireland, the Annual 23rd Conference of the Section of Bioengineering of the Royal Academy of Medicine in Ireland. Queen’s University, Belfast, Northern Ireland. (Presentation).


Aprile P., Kelly D.J., 2019. *Influence of substrate mechanical properties and cell morphology on chondrogenesis of mesenchymal stem cells within interpenetrating network hydrogels.* Winter School ROAD network, Paris, France. (Poster)

Chapter 1  Introduction

1.1  Mechanobiology of MSC

It is well established that matrix stiffness and topography have a profound impact on cell behaviour for numerous different cell types, including muscle stem cells\textsuperscript{10}, endothelial cells\textsuperscript{11} and mesenchymal stem cells \textsuperscript{5,12}. Mechanobiology is an emerging field that combines the tools of biologists and engineers with the specific aim of understanding how molecules, cells, tissues and organs can sense and respond to their local mechanical environment. A central element of mechanobiology is \textit{mechanosensing}. It is known that cells can experience the elasticity of the local environment by exerting traction forces, however what is still unclear is how cells transduce this information into a cellular response\textsuperscript{13}.

2D substrates have been broadly used to study mechanotransduction mechanisms, but there is increasing evidence that cell morphology, cell-cell and cell-matrix interactions are considerably dependent on dimensionality (i.e. culture of cells on a 2D substrate or encapsulation into a 3D matrix). Therefore new 3D biomaterial systems are required that are capable of mimicking key aspects of the \textit{in vivo} cellular environment\textsuperscript{14}. Such systems should allow for the independent control of cell shape and substrate stiffness in order to engineer environments supportive of specific cellular phenotypes.
1.2 Hydrogel to tune the cellular environment.

Hydrogels are commonly used as scaffold in tissue engineering and as tuneable environments in the field of mechanobiology. Among all the synthetic and natural polymers that can be used for this purpose, polysaccharides represent a class of macromolecules of particular interest for their abundance, variety of composition and chemical adaptability\textsuperscript{15}. A key challenge when designing new biomaterials for use in mechanobiology and tissue engineering is the capability to temporally control the local cellular environment. In recent years there has been increased interest in the use of Interpenetrating Polymer Network (IPN) hydrogels as smart systems capable to both entrapping cells and responding to physiological triggers at the appropriate time and site of action\textsuperscript{16}. These IPN often show physico-chemical properties that can be different from those of the macromolecular constituents. They can be designed to be cell compatible, bioadhesive, and stimuli responsive. In particular, IPN incorporating alginate have been used for cell encapsulation because of its high versatility and tailorable mechanical properties. It has been demonstrated that alginate can be easily combined with other natural or synthetic polymers to develop IPN capable of modulating cell morphology and/or substrate stiffness\textsuperscript{17,18}.

Bone marrow derived mesenchymal stem cells/marrow stromal cells (MSC) could potentially provide an effective cell source for tissue engineering and regenerative medicine applications. MSC can differentiate along several pathways including the chondrogenic, osteogenic, adipogenic and myogenic lineages. The ability to direct MSC differentiation in a stable and concerted manner is crucial to the success of tissue engineering and regenerative medicine therapies. Recent in vitro observations pointed out that MSC can probe and modify their pericellular niche and the bi-directional interplay of these interactions play a role in MSC fate determination\textsuperscript{19}. As mentioned before, matrix stiffness and cell shape modulate the
differentiation of MSC towards distinct lineages. When seeded onto a 2D substrate, depending on the soluble factors in the medium, MSC have been shown to adopt an adipogenic\textsuperscript{12,20} or chondrogenic\textsuperscript{21} phenotype when cell size is restricted or when contractility is reduced. Conversely, spreading and high contractility promotes osteogenic\textsuperscript{12,22} and myogenic differentiation\textsuperscript{21}, albeit through different mechanotransduction pathways. Recently, cytoskeletal tension and cell geometry have been found to influence the activity of YAP/TAZ transcription factor\textsuperscript{23}. YAP/TAZ is inhibited when cells are confined to small adhesive areas. In contrast, when cells are seeded on large micropatterned areas, which enable cell spreading and large adhesive areas, YAP/TAZ becomes nuclear and active\textsuperscript{24}.

3D culture environments have been shown to promote alternative behaviours to that observed in 2D. For example, in covalently crosslinked hyaluronic acid hydrogels, it has been demonstrated that MSC differentiation is directed by the generation of degradation-mediated cellular traction, independently of cell morphology or matrix stiffness\textsuperscript{25}. Other mechanical factors can also regulate MSC fate following encapsulation into 3D hydrogels. It has been demonstrated that MSC differentiation in a RGD-modified alginate hydrogel is dependent on the viscoelastic characteristics of the material. Increases in MSC spreading, proliferation, and osteogenic differentiation were observed in hydrogels with faster relaxation properties. Moreover, even in soft gels, as the stress relaxation behaviour of the gel increases, an inhibition of adipogenesis was observed. This is believed to occur as a fast matrix stress relaxation mediates integrin adhesion, actomyosin contractility and nuclear localization of YAP\textsuperscript{7}.

Cellular forces can be modulated by many effectors, such as myosin II, myosin light chain kinase, Rho/ROCK pathway and actin polymerization. It is well known that Rho family small GTPases can regulate cell shape through modulating
cytoskeleton and vice versa. Using a 2D system of micropatterned substrates, it has been shown that the switch from MSC adipogenesis to osteogenesis is driven by RhoA/ROCK\textsuperscript{12}, while the switch from chondrogenesis to myogenesis depends on the activation of Rac1\textsuperscript{21}.

In addition to cues generated in response to alterations in the stiffness and composition of the extracellular matrix (ECM) \textit{in vivo}, stem cells will also be exposed to extrinsic mechanical loading which is known to influence their ultimate fate. The type (i.e. compression, fluid flow, tension, hydrostatic pressure), frequency, magnitude, and duration of loading all affect MSC lineage commitment. Hydrostatic pressure (HP) is a key regulator of chondrogenesis\textsuperscript{26}, however the exact mechanism by which it exerts its influence on MSC is unknown. It has been proposed that increased HP can lead to depolymerisation of cytoskeletal polymers into free monomers, therefore disrupting stress fibre assembly, interfering with the cellular force transmission machinery\textsuperscript{27–30}. Understanding how physiological levels of extrinsic mechanical cues regulate the fate of MSC will be integral to releasing their full therapeutic potential.

1.3 Objectives of the thesis.

Despite strong evidence that mechanical cues regulate chondrogenesis of stem/progenitor cells, we have little insight into the mechanisms by which cells sense and integrate this information during differentiation. This thesis seeks to combine developments from the fields of biomaterials, mechanobiology, molecular analysis and microscopy to investigate how extrinsic and intrinsic mechanical stimuli regulate chondrogenesis of MSC. Examining the role of such mechanical stimuli in regulating stem cell chondrogenesis is fundamental not only because it
aids our knowledge of a complex developmental process, but also because of its implication in regenerative medicine. The appropriate application of biophysical cues may help to engineer environments *in vitro* that mimic the conditions that exist within a joint during articular cartilage development. This thesis will test the hypothesis that chondrogenesis of MSC is regulated by both intrinsic (specifically matrix stiffness and initial cell shape) and extrinsic (cyclic HP) mechanical cues. In doing so, this thesis seeks to identify specific combinations of mechanical stimuli that will support or suppress chondrogenesis of MSC. The specific aims of this thesis are to:

1. Develop a collagen and alginate IPN hydrogel to study the mechanobiology of cells in 2D and 3D environments, where both substrate stiffness and cell morphology can be independently tuned (see Figure 1-1). Chapter 4 and 5 will describe in details the biomaterial fabrication process of the 2D and the 3D IPN system respectively.
Figure 1-1 | Regulation of matrix stiffness and cell morphology through alginate crosslinking in the IPN. Schematic of alginate crosslinking depicting in a) the ionic crosslinking of the polymer. b) Modulation of matrix stiffness. c) Cell morphology can be tailored by delaying the crosslinking of the alginate network. The immediate crosslinking of the alginate (i) locks the cells to a rounded morphology, while a (ii) delayed crosslinking allows the cells to spread over the collagen fibres.

2 Determine the influence of substrate stiffness on the chondrogenic differentiation of MSC seeded onto the surface of IPN hydrogels (2D culture; see Figure 1-2a). The expression of chondrogenic genes and the
cellular localization of specific signalling molecules will be examined in MSC seeded at different densities into soft and stiff substrates (Chapter 4).

3 Explore how chondrogenesis of MSC is influenced by both substrate stiffness and cell morphology following encapsulation into IPN hydrogel (3D culture; see Figure 1-2b). The initial cell morphology and substrate elasticity that best supports chondrogenesis will be determined, and the mechanism by which this occurs will be explored (Chapter 5).

4 Mimic the mechanical environment of a loaded knee joint by applying physiological magnitudes of HP to MSC encapsulated within the IPN hydrogel system. The influence of applying HP before or after TGF-β3 induced differentiation will be assessed (see Figure 1-2c). The mechanism by which the temporal application of extrinsic mechanical cues regulates chondrogenesis of MSC will be explored (Chapter 6).
Figure 1-2 | Schematic of experiments summary. a) In Chapter 4 will be explored the IPN system and the effect of cells interaction and substrate stiffness on MSC grown on 2D IPN. b) Chapter 5 will to examine the (i) effect of dimensionality on MSC grown on or into IPN and (ii) the interplay of matrix stiffness and cell morphology on MSC chondrogenesis. c) Finally, in Chapter 6 will be discussed the application of HP at different stages of MSC chondrogenic differentiation in presence or absence of TGF-β3.
Chapter 2  Literature review

In this chapter a background to this work is provided through a review of the applicable scientific literature. Firstly, the developmental processes of joint and articular cartilage formation are introduced. The role of biophysical cues as regulators of the lineage commitment of adult mesenchymal stem/progenitor cells is then reviewed. Following this, the molecular mechanisms underlining MSC mechano-sensation are presented through a description of candidate pathways. Finally, the role of extrinsic mechanical stimulation, and in particular hydrostatic pressure, on chondrogenic commitment is outlined to better understand how joint specific mechanical stimuli influence MSC fate.
2.1 Development of synovial joint formation

Developmentally the cells that ultimately form the synovial joints are derived from the mesoderm. Mesoderm (the middle of the three embryonic germ layer\(^{31}\)) precursor cells are molecularly specified at the blastula stage (during this stage the embryo undergoes cleavage to become multicellular). Next, gastrulation occurs generating an embryo with three germ layers\(^{32}\). The mesoderm precursors mature into mesoderm cells through the epithelial to mesenchymal transition (EMT) process. These cells ingress from the epiblast through the primitive streak and migrate between the ectoderm and endoderm germ layers in a spatially and temporally ordered manner\(^ {33}\). The mesoderm layer is configured as an axial, paraxial, intermediate, lateral plate mesoderm (LPM) and an extraembryonic mesoderm (Figure 2-1). However, only the axial, paraxial and LPM are able to generate skeletal elements during development. Cells from the splanchnic layer of the LPM contribute to nearly the entire cardiovascular system, while the somatic area of the LPM give rise to all distal skeletal elements: bone in the limbs, pelvic girdle, sternum and part of the shoulder girdle\(^ {34}\).
Figure 2-1 | Schematic of vertebrate early development of mesoderm formation and patterning. A) EMT and migration of mesoderm precursor in the primitive streak (PS). B) Embryonic germ layers. IM: intermediate mesoderm; ExEM: extraembryonic mesoderm; NT: neural tube; NC: neural crest34.

Limb skeletogenesis initiates with the formation of a small bud of mesenchymal cells covered by ectoderm, which will condensate in a Y-shaped pattern35. Joint formation is firstly characterized by the emergence of a mesenchymal interzone, described by stellate-shaped cells embedded in a loose matrix36. The interzone displays a packed intermediate cell layer and two outer cell layers facing the distal and proximal end of the long bone anlage. This cartilaginous template will generate the future bone through a defined sequence of events involving hypertrophy and replacement by bone (endochondral ossification, see Figure 2-2). This process take place proximally and distally in the anlage with a growth plate remaining at the epiphyses for continued elongation.
Figure 2-2 | Schematic of endochondral ossification. The cartilage anlage (1) starts to cavitate and calcify, the bone collar forms around the diaphysis (2). The periosteal bud invades the internal cavities; the diaphysis elongates and a medullary cavity forms (3). The growth plate remains at the epiphyses and spongy bone forms (4).

The synovial joint, which is located at the interface of the rudiments, is composed of a chondrogenous layer on the developing bone surfaces and the intermediate layer where joint cavitation will later take place. At birth, most of the cartilage template is replaced by mineralised bone with the exception of growth plates, which will persist as a source of immature chondrocytes for elongation of the long bone until the organism reaches its adult size. Permanent articular cartilage persists at the ends of the long bones at the point where it caps the epiphyses to reduce friction at joint articulations.
2.2 Role of N-cadherin mediated cell-cell adhesion during chondrogenic differentiation

Chondrogenesis is influenced by many extra- and intracellular signaling pathways, in particular a hallmark of cartilage formation is represented by cell condensation. Cellular condensation was first described by Fell in 1925 in the avian embryonic limb. These cell aggregates were associated with an increase in cell-packing density and absence of proliferation. Cell-cell adhesion proteins such as N-cadherin (NCAD), mediate cellular condensation in the regions destined to become cartilage.

N-cadherin (NCAD) is a Ca\textsuperscript{2+} dependent single transmembrane glycoprotein belonging to the classic cadherin group (Figure 2-3). All members of the cadherin superfamily are characterized by a unique domain, called cadherin motif or EC domain, containing the negatively charged DXD, DRE, and DXNDNAPXF amino acids sequence motifs, which are involved in Ca2+ binding. Although the presence of the EC domains is the hallmark of this molecular family, the amino acid sequences of the cytoplasmic domain, significantly diverge among the members, suggesting that their functional diversification has occurred during evolution. The classic cadherins group mediates cell-cell adhesion by homotypic protein-protein interaction through their extracellular domain, while binding the actin cytoskeleton and the transcription factor β-catenin with the cytoplasmic domain.
Figure 2-3 | Schematic diagram of the molecular structure of the cadherin superfamily from Yagi et al.\textsuperscript{38}. Protein depicted in blue and cytoplasmic interactions in yellow and/or pink.

In limb buds of chick embryo, although NCAD shows a scattered expression pattern in the core region in the pre-cartilage stage, its presence increases as condensation begins and diminishes as chondrogenic differentiation begins. As cartilage development progresses, cell–cell interactions progressively diminish as progenitor cells differentiate, and subsequently deposit and interact with increasing levels of ECM in the microenvironment. Indeed, mature cartilage is devoid of NCAD, whereas the cells in the forming perichondrium surrounding the developing cartilage still exhibit high levels of NCAD\textsuperscript{41}. The spatiotemporal regulation of N-cadherin during chondrogenesis suggests an important modulatory role of cell-cell interaction and downstream regulation. During development, cell surface metalloprotease ADAM10 regulate NCAD by cleaving the extracellular domain, thereby taking control of β-catenin membrane-bound and cytoplasmic pool levels.
which in turn regulate gene expression\(^4^2\). \(\beta\)-catenin stimulates chondrocyte hypertrophy and endochondral ossification in developing limbs. The cleavage of N-cadherin at the extracellular domain produces an ectodomain soluble fragment which can act as extracellular signaling molecule, and NCAD/C-terminal fragment 1 (CTF1), a membrane fragment (Figure 2-4). Then, presenilin1 (PS1)/\(\gamma\)-secretase-mediated cleavage of NCAD at the intracellular domain produces NCAD/C-terminal fragment 2 (CTF2). This last cleavage promotes the nuclear translocation of \(\beta\)-catenin and leads to enhanced transcriptional nuclear signaling\(^4^3\).

**Figure 2-4 | NCAD cleavage sites\(^4^2\).**

In the last years, many studies focused on how to tether NCAD properties to recapitulate the chondrogenic differentiation for cartilage tissue engineering purposes. For instance, the incorporation of NCAD-derived peptides (i.e. HAV) that mimics cell-cell interaction allowed to understand the timing and duration of NCAD presentation during MSC chondrogenic differentiation. In particular, hyaluronic acid hydrogels (HA) functionalized with HAV resulted in dose-dependent increase in early chondrogenesis of embedded MSC and consecutive matrix deposition\(^4^4\). While the introduction of an ADAM 10-cleavable domain abolished the increase in chondrogenic gene expression and matrix production\(^4^5\).
The diverse cell adhesion interaction during the chondrogenic differentiation of MSC influences their mechanobiological mechanism. In a 2D system of HA hydrogels of different stiffness, the ligation of HAVDI adhesive domains (from NCAD EC1 domain) led to an alteration of the intrinsic force-sensing mechanism. The presence of HAVDI domains correlated with a reduced YAP/TAZ nuclear translocation and to a Rac1-GTP-dependent reduction of myosin IIA into focal adhesions (FAs). YAP/TAZ cytosolic sequestration inhibited cell proliferation and RUNX2 expression, while the reduced presence of myosin IIA hindered the maturation of the adhesions with increasing substrate stiffness, as such that cell contractile forces were limited\(^9\). Altogether these studies suggest the beneficial effect in harnessing NCAD properties for tuning cellular response for cartilage regeneration approaches.
HAVDI ligation influences MSC mechanosensing. The increase of cell-cell contact (through HAVDI/NCAD interactions) reduces the contractile state of the cell by inhibiting Rac1-GTP levels and therefore limiting myosin IIA incorporation into FAs and inducing YAP/TAZ cytosolic sequestration. In this scenario, cells on intermediate stiffness (15 kPa) behaves as if they were on a much softer substrate (6 kPa) with only ECM ligands.

2.3 Mesenchymal stem cells

In the early 1970s, Friedenstein et al. described a heterogeneous population of bone marrow derived cells, different from the hematopoietic stem cells, capable of limited mutual transformation. These cells were found to have the potential for osteogenesis. Caplan described these cells as Mesenchymal Stem Cells (MSC). MSC derived from human and mammalian bone marrow and periosteum, could be expanded in culture while maintaining their in vitro capacity to be induced to generate a variety of mesodermal tissues through the so called ‘Mesengenic Process’.
Recent studies revised the notion of the MSC, hypothesizing that local stem/progenitor cells, such as skeletal stem cells, are perivascular cells that are formed by a developmental process shared by connective tissues. During development, as blood vessels invade tissue, they capture local cells that are committed to a lineage and incorporate them in a mural or subendothelial position, giving the blood vessels stability (Figure 2-6). These captured cells remain quiescent until liberated from the blood vessel wall owing to an injury or tissue turnover, at which point they help reform the cell types of that tissue. This idea would explain why skeletal progenitors are perivascular in the bone marrow, some perivascular cells in skeletal muscle are myogenic and adipocyte progenitors in adipose tissue are perivascular. Thus, it is now more commonly accepted that there isn’t a common MSC for all connective tissues. For instance, at least three embryonal sources are able to generate bone tissue: neural crest (facial bones), paraxial mesoderm (axial bones) and somatic lateral plate mesoderm (appendicular bones). Although it is not clear yet which nomenclature would best represent the biological meaning of this cell population, this thesis will refer to bone marrow derived mesenchymal stem cells/marrow stromal cells (MSC) as a heterogeneous population of cells that contains tissue specific progenitor cells (i.e. skeletal stem cells).
Figure 2-6 | Schematic of the formation of MSC in the bone marrow. During development, the ingrowing blood vessel invades the primitive bone marrow stroma recruiting cells from the outer layer (perichondrium and periosteum). EC: endothelial cell, HSC: hematopoietic stem cell. Adapted from Bianco et al.49.

Recent in vitro studies have described how the bi-directional interplay of MSC mechanical environment and their own secreted pericellular matrix influences cell differentiation19. More in general, environmental cues determine the lineage commitment of MSC, with substrate stiffness8,52,53, cell morphology12,21, oxygen tension54–56, mechanical forces57,58 and biochemical cues59–61 all playing pivotal roles in fate determination. The role of some of these biophysical cues will be discussed later in this review. Unravelling the mechanism by which such factors regulate MSC differentiation could lead to the development of novel tissue engineering strategies.
2.4 The role of cell shape on MSC differentiation.

Cells can adapt their morphology to fulfil determined functions. For instance, in order to deliver signals rapidly over a long distance neurons developed long axons, while adipocytes assume a rounded morphology to maximize lipid accumulation. Embryonic development and tissue regeneration often implicate changes in cell morphology which can influence tissue function and structure\textsuperscript{6,62}. Many studies focusing on patterning the morphology of MSC support the hypothesis of cell shape as regulator of stem cell fate.

McBeath et al.\textsuperscript{12} used micro-contact printing to coat surfaces of polydimethylsiloxane (PDMS) with adhesive ECM islands of different areas to either confine or induce cell spreading. By combining this system with the supplementation of a bi-potential differentiation medium able to induce both osteogenic and adipogenic differentiation, they demonstrated that cells allowed to spread underwent osteogenesis, while less spread, more rounded cells became adipocytes.

The authors postulated that the process was controlled by cytoskeletal tension, specifically through the RhoA and ROCK signalling pathways. RhoA is a key regulator of contractility, while ROCK is a Rho effector involved in myosin contraction. The mechanism by which RhoA affects lineage commitment was examined using MSC which were modified to overexpress or inhibit RhoA. Firstly, when cell morphology was not modified through their micro-patterning technique and in absence of differentiation factors, the overexpression of RhoA increased the expression of osteogenic markers, while the inhibition of RhoA increased the expression of adipogenic markers. Similarly, in presence of osteogenic media MSC underwent adipogenesis when RhoA was inactivated, while a costitutively active RhoA induced osteogenesis in cells cultured in adipogenic media. When RhoA was
overexpressed in MSC forced to adopt a round morphology by their local topography, MSC continued to undergo adipogenesis. Similarly, when RhoA was inhibited in MSC forced to adopt a spread morphology, they continued to express markers of osteogenesis. These results imply that soluble factor signalling can be overcame by RhoA activity and although RhoA signalling is modulated by cell shape, it is not necessarily the driving factor by which changes in cell shape determine MSC lineage commitment (Figure 2-7). The role of ROCK in the process was then examined by either inhibiting or overexpressing it in MSC forced to adopt either a round or spread morphology. In contrast to RhoA, when ROCK was inhibited, both round and spread cells expressed adipogenic markers. Similarly, when ROCK was upregulated, both round and spread cells expressed markers for osteogenesis. These results demonstrate the pivotal role played by cytoskeletal tension in controlling MSC differentiation (Figure 2-7).

Figure 2-7 | Model of mechanically mediated lineage commitment to an adipogenic or osteogenic fate

12
Gao et al. further investigated how cell shape confers a switch between a chondrogenic and a smooth muscle cell (SMC) fate in MSC. They used the same system of micropatterned substrates reported by McBeath et al, but instead maintained MSC in a bi-potent medium containing TGF-β3 able to induce both chondrogenic and smooth muscle differentiation. Chondrogenesis was observed when MSC were prevented from spreading, whereas spread cells underwent SMC differentiation. To confirm the involvement of cytoskeletal tension, the authors investigated the role of the small GTPases family in modulating the effect of TGF-β3 signalling in round and spread cells (Figure 2-8). Unlike previous experiments exploring the role of cell shape in the osteogenic-adipogenic fate decision, RhoA activity didn’t vary among the different conditions. On investigating the role of other GTPases, Rac1 activity was found to be significantly higher in spread cells. Inducing a constitutively active form of Rac1 in spread cells induced SMC differentiation in the absence of TGF-β3, while a dominant negative form of Rac1 inhibited SMC differentiation in the presence of TGF-β3, demonstrating the fundamental role of Rac1 for SMC lineage commitment. Moreover, increased activity of Rac1 inhibited chondrogenesis of MSC cultured in pellets, while decreasing its activity had no effect on chondrogenic differentiation.
2.5 The role of substrate stiffness on MSC differentiation.

Early studies from Engler et al.\textsuperscript{5} demonstrated the sensitivity of MSC to tissue elasticity. Their hypothesis was based on the fact that many of the tissues which MSC migrate to (such as bone, smooth muscle and neurological tissue) are synthesised by cells which have an anchorage dependency\textsuperscript{5}. Different cell types respond to tissue matrix stiffness, altering their cytoskeleton and focal adhesion structures resulting in a modification of their morphology, transcript profile and marker proteins\textsuperscript{63–65}. Matrix sensing requires the ability to pull against the matrix; it is thought that the nonmuscle myosin II isoforms (NMM IIA, B, C) are implicated in tensioning actin structures, which are in turn connected to focal adhesions (FA), transmitting forces from inside the cell to the ECM. In contrast, the application of an external force to the cell-ECM unit leads to structural deformations and rearrangements of the ECM, force transmission through the FA, and deformation of nearly every aspect of intracellular structure, including the position of mitochondria, endoplasmic reticulum, and the nucleus\textsuperscript{66}. To test the
implication of matrix elasticity in MSC fate determination, Engler et al.\textsuperscript{5} cultured human mesenchymal stem cells (hMSC) onto polyacrylamide gels of variable stiffness, coated with collagen type I. They found that a soft substrate which mimicked the stiffness brain tissue (0.1 – 1 kPa) promoted the expression of genetic markers and had a dendritic morphology indicative of a neurological lineage \textsuperscript{5}. Similarly, gels which had a stiffness representative of smooth muscle (8 – 17 kPa) promoted myogenesis and a spindle shaped morphology\textsuperscript{5}. Finally stiff gels mimicking the elasticity of osteoid (25 – 50 kPa) promoted a polygonal osteoblastic morphology and an increase in osteogenic markers\textsuperscript{5} (Figure 2-9).

![Figure 2-9](image)

**Figure 2-9 | MSC protein expression profile is elasticity dependent.** The neuronal marker β3 tubulin is expressed only on the soft matrices. The muscle marker MyoD1 is upregulated and nuclear localized on firm matrices. The osteoblast transcription factor CBFα1 is express only on stiff gels\textsuperscript{5}.

Although cells encounter biochemical and physical cues simultaneously, the mechanisms by which this information is assimilated to direct cellular responses
remain unclear. It has been shown that physical cues can alter the cellular response to growth factors\textsuperscript{21,67}. In particular the work from Allen \textit{et al.}\textsuperscript{68} demonstrated that cartilage-like substrate stiffness (0.5 MPa) enhanced chondrocytes gene expression and primed cells for a robust response to the transforming growth factor (TGF)-\(\beta\), which plays a major role in the regulation of chondrogenesis. Their work showed that ECM stiffness regulated chondrocytes differentiation by promoting ROCK-mediated autocrine TGF-\(\beta\)1 expression on substrates softer than plastic (1.1, 0.5 and 0.2 MPa). In particular, primary chondrocytes and the chondrogenic cell line ATDC5 had an increase in Smad3 phosphorylation (p-Smad3) when cultured on 0.5-MPa substrate. Their data suggested a stiffness-specific mechanism by which the activity of the autologous TGF-\(\beta\)1 was responsible for the increase in p-Smad3 and \textit{COL}2 expression. The combinatorial effect of substrate stiffness (0.5-MPa) and TGF-\(\beta\) supplementation caused a striking increase of p38 phosphorylation in ATDC5 cells. The inhibition of p38 activity caused a 2.5-fold decrease of \textit{COL}2 expression when cells were grown on 0.5MPa substrates alone, while it was responsible for a 5-fold repression of \textit{COL}2 when these cells were also supplemented with TGF-\(\beta\) depicting a p38 MAPK-dependent synergistic induction of chondrocytes gene expression. The range of stiffness present in cartilage varies spatially and temporally, such that each may have unique instructive roles. Consistent with the stiffness of adult articular cartilage, precommitted chondrocytes (ATDC5 or primary chondrocytes) showed maximal chondrocyte gene expression on 0.5-MPa substrates. More compliant matrices may be more chondroinductive during lineage selection, in fact Park \textit{et al.}\textsuperscript{69} showed that the induction of chondrogenic gene expression in MSC occurs on compliant 1 kPa substrates. The same study confirmed that matrix stiffness is able to determine the smooth muscle,
chondrogenic and adipogenic differentiation of MSC only when in synergy with TGF-β.

Despite many studies tried to unravel the role of substrate stiffness in chondrogenesis, efforts have been also addressed to examine this role in a more physiologically relevant environment. Of particular interest is the work of Huebsch et al.\textsuperscript{70} in which they studied the effect of 3D microenvironments of different rigidity on mouse MSC (mMSC) fate determination. They found that a softer 3D microenvironment (2.5-5 kPa) promoted an adipogenic phenotype, while osteogenic commitment was mainly occurring at intermediate rigidity (11-30 kPa). In order to investigate potential underlying mechanisms, they investigated the adhesion dynamics of mMSC within the hydrogel.

Cells were shown to be able to reorganize RGD domains of the hydrogel by pulling on the local matrix. Using FRET they could measure the traction-mediated clustering of RGD ligands, which was also dependent on the stiffness of the matrix and peaked at 22 kPa, strongly correlating to the matrix stiffness most supportive of osteogenic commitment (Figure 2-10)\textsuperscript{70}. Based on these findings they concluded that MSC can interpret mechanically diverse substrates as having different adhesion-ligand presentations, thus cell-traction forces could be exploited to mechanically tune synthetic materials in order to direct cell fate determination in a 3D environment.
Figure 2-10 | Matrix compliance determines MSC fate in 3D matrix culture.

In situ staining for ALP activity (osteogenic biomarker, blue) and neutral lipid accumulation (adipogenic biomarker, red)\(^8\).

It is known that the crosslinking density of a material can impact its physical properties, including mechanical stiffness and macromolecular diffusivity. Bian et al.\(^71\) demonstrated that varying the crosslinking density of 3D hydrogels composed of hyaluronic acid (HA) by means of macromer concentration or extent of exposure time to ultraviolet light (UV), it was possible to modulate human MSC chondrogenic differentiation. In particular, a higher crosslinked gel which resulted in a stiffer matrix and lower diffusivity, resulted in an overall decrease of cartilage matrix production and the promotion of a more hypertrophic phenotype. Increased \(COLX\) and \(ALP\) mRNA expression, and higher matrix calcification, were observed in MSC embedded into stiffer gels\(^71\). The inhibition of myosin II and ROCK had no effect on MSC hypertrophy and calcification, indicating that the hypertrophic differentiation within the stiffer gel wasn’t related to the generation of cell actomyosin tension.

Although there are many studies on the effect of hydrogel stiffness on cartilage matrix formation by chondrocytes, the impact of hydrogel viscoelasticity on chondrocytes remains less explored\(^72\). In viscoelastic hydrogels stresses are relaxed over time and exhibit creep. Lee et al.\(^73\) used alginate of different molecular weights to obtain a range of viscoelastic hydrogels of same stiffness (3D culture). They found that slower relaxing hydrogels could block the volume expansion of the
chondrocytes embedded and promote the secretion of interleukin-1β (IL-1β), leading to the upregulation of cartilage matrix catabolic genes (ADAMTS4, MMP13) and ultimately to cell death. An important finding of Lee et al.\textsuperscript{73} work was that cell sensing of hydrogels viscoelastic properties was occurring in an adhesion-independent manner, since their alginate hydrogels system lacked of RGD binding sites. They demonstrated that chondrocytes can utilize changes in volume to sense the viscoelastic properties of their niche (see Figure 2-11), however it cannot be excluded that a system of adherent cells might use a combination of adhesion-independent and adhesion-mediated mechanisms to sense matrix viscoelasticity.

\textbf{Figure 2-11 | Volume restriction regulates chondrocytes phenotype.} In a slow relaxing hydrogel, elastic stresses resist the volume expansion causing IL-1β production, catabolic genes upregulation and cell death. When the elastic stresses are dissipated over time (fast relaxing hydrogels), the cell can expand its volume and promote an interconnected cartilage matrix.
2.6 The role of YAP/TAZ in mechanotransduction.

Studies exploring the transduction of mechanical signals in different cell types have identified YAP (Yes-associated protein) and TAZ (transcriptional coactivator with a PDZ-binding domain) as key mechanosensing molecules\textsuperscript{24}. YAP and TAZ are two related transcriptional coactivators known as mediators of the Hippo signalling pathway, regulating organ growth and tumorigenesis, cell proliferation, differentiation and epithelial stemness\textsuperscript{74}.

The core components of the Hippo pathway comprise a regulatory serine–threonine kinase module and a transcriptional module (Figure 2-12)\textsuperscript{75}. The kinase module comprises two groups of kinases: mammalian STE20-like protein kinase 1 (MST1) and MST2 and large tumor suppressor 1 (LATS1) and LATS2 in combination with their activating adaptor proteins Salvador family WW domain-containing protein 1 (SAV1) and MOB kinase activator 1A/B (MOB1A/B), respectively\textsuperscript{75}. The transcriptional module comprises the transcriptional coactivators YAP and TAZ and several transcription factors, of which the most prominently studied are TEA domain family members 1–4 (TEAD1–4)\textsuperscript{75}. YAP and TAZ do not contain any DNA-binding domains and thus bind to and elicit their transcription activation via TEADs\textsuperscript{75}. Nonetheless, YAP and TAZ do utilize other transcription factors, such as SMADs, p63, RUNX2, and PAX, which diversifies the transcriptional output of the Hippo pathway\textsuperscript{76}. In a classical view, when the upstream kinase module is activated, MST1/2 phosphorylates SAV1, MOB1A/B, and LATS1/2 to activate the LATS1/2 kinases, which in turn directly phosphorylate YAP and TAZ on multiple serine residues leading to cytoplasmic retention of YAP/TAZ via a 14-3-3 interaction. Furthermore, the phosphorylation of YAP/TAZ by LATS1/2 primes YAP/TAZ for proteasomal degradation. By contrast, when the
kinase module is inactivated, hypophosphorylated YAP/TAZ translocate into the nucleus and induce target gene expression (Figure 2-12)\textsuperscript{75}. 
The core of the Hippo pathway\textsuperscript{75}. When the upstream kinase module is activated, MST1/2 phosphorylates SAV1, MOB1A/B, and LATS1/2 to activate the LATS1/2 kinases, which in turn directly phosphorylate YAP and TAZ on multiple serine residues leading to cytoplasmic retention of YAP/TAZ via a 14-3-3 interaction. Furthermore, the phosphorylation of YAP/TAZ by LATS1/2 primes YAP/TAZ for proteasomal degradation. The requirement for MST1/2 to phosphorylate LATS1/2 is cell-type dependent, suggesting the existence of additional kinases (drawn as a question mark) that regulate the activating phosphorylation of LATS1/2. By contrast, when the kinase module is inactivated, hypophosphorylated YAP/TAZ translocate into the nucleus and induce target gene expression.

The Hippo pathway is integrated with TGF-β and BMP signalling on multiple levels\textsuperscript{77,78}. In human embryonic stem cells, impairment of the nuclear translocation of TAZ results in the sequestration of a complex comprising TAZ,
SMAD2, and SMAD3 in the cytosol\textsuperscript{77}. On activation, nuclear YAP/TAZ form a complex with SMAD2/3 to bind TEADs or OCT4, which mediates pluripotency\textsuperscript{79}. On loss of YAP/TAZ, these stem cells lose pluripotency potential and differentiate\textsuperscript{79}.

Of particular interest, low YAP/TAZ activity promotes adipogenesis, while high YAP/TAZ activity drives MSC towards osteogenesis\textsuperscript{24,80,81}. The role of YAP/TAZ in chondrogenesis of MSC has been explored by the work of Karystinou \textit{et al.}\textsuperscript{82}, who examined whether low nuclear YAP is required for chondrogenic differentiation. To this end they overexpressed either wild- type or a non-phosphorylatable mutant form of YAP in mouse C3H10T1/2 MSC and subjected the cells to chondrogenic culture conditions. Forced YAP expression decreased chondrogenic differentiation, while mutant YAP almost completely abrogated chondrogenesis\textsuperscript{82}. In this work Karystinou demonstrated that high YAP activity impairs chondrogenesis at least in part through suppressing Smad signalling. Finally, to determine YAP involvement with the regulation of chondrogenesis \textit{in vivo}, they analysed YAP and pYAP (phosphorylated YAP) abundance during mouse embryonic limb development (Figure 2-13)\textsuperscript{82}. While YAP was mostly nuclear in cells in the perichondrium, cells of the cartilage anlage showed high levels of phosphorylated, cytosolic YAP. These findings are consistent with the previous \textit{in vitro} observations that YAP inhibits chondrogenesis. Of note, YAP expression was increased again during chondrocyte terminal differentiation, suggesting that YAP may play a role in chondrocyte hypertrophy\textsuperscript{82}. 
**Figure 2-13 | Yap and pYap expression during limb development.**

Consecutive histological sections from developing hindlimbs of mouse embryos at E13.5 in A, E14.5 in B, E16.0 in C. Boxed area on left shows approximate location of higher magnification images on right. P: perichondrium, C: cartilage anlage. HC: hypertrophic cartilage. Bars indicate 100 μm (tile scan images on left; 20× objective) and 10 μm (higher magnification images on right; 100× oil objective)\(^8\).  

In contrast to the work of Karystinou et al.\(^8\), Deng et al.\(^9\) found that YAP1 promotes early chondrocyte proliferation but inhibits chondrocyte hypertrophy and maturation both *in vitro* and *in vivo*. The authors examined the endogenous expression pattern of YAP1 in the long bones during endochondral bone formation. YAP1 was expressed in resting and proliferative chondrocytes but less in hypertrophic chondrocytes at the embryonic stage E14.5 (Figure 2-14A). pYAP1
was strongest in the hypertrophic chondrocytes where COLX and RUNX2 expression were detected (Figure 2-14A), suggesting a lower activity of YAP1 during chondrocyte maturation. *In vitro* experiments with limb buds micromasses showed an increasing expression of chondrocyte markers from day 5 to day 7, RUNX2 picked at day 10 (Figure 2-14B). YAPI expression was gradually reduced along with cartilage nodule formation (Figure 2-14C), while pYAP1 was increased.
Figure 2-14 | Decreasing expression of YAP1 during chondrocyte differentiation. A) Yap1 was expressed in the growth plates of wild-type tibias at E14.5 as shown by immunofluorescence (RZ, resting chondrocytes; PZ, proliferative chondrocytes; PHZ, prehypertrophic chondrocytes; HZ, hypertrophic chondrocytes). Scale bars, 100 mm. B and C) Gene expression analysis of micromass culture.

Homozygous transgenic mice overexpressing YAP1 in chondrocytes and osteoprogenitor cells displayed a smaller skeleton size than that of the wild-type or the heterozygous, while YAP1 knockdown produced a bigger skeleton size than that of the age-matched wild-type littermates. In this study, bone repair was remarkably impaired and most of the chondrocytes were maintained at an immature state in the cartilaginous callus tissues.
The scientific community agrees in YAP’s role as mechanosensor, however the controversial findings highlighted from the literature doesn’t allow for a unique interpretation of YAP’s role during chondrogenesis of MSC.

2.7 Interpenetrating network hydrogels

Classical culture systems are typically 2D and although their simplicity of use, cells cultured onto these platforms tend to assume aberrant behaviors: de-differentiation, flattened shape and altered response to drugs are only some examples. Biomaterial systems have been developed to mimic the biological milieu, thus bridging the gap between conventional cultures and complex native in vivo contexts. Hydrogels - water-swollen network of polymers - represent a valid alternative for cell growth since they can mimic or incorporate components of the native ECM and have tunable mechanical properties. In particular, Interpenetrating Polymeric Networks (IPNs) have emerged as innovative biomaterial to help understanding how ECM mechanics regulate cell behavior. The IUPAC defines an IPN as: “a polymer comprising two or more networks which are at least partially interlaced on a molecular scale but not covalently bonded to each other and cannot be separate unless chemical bonds are broken.” The combination of the properties of each constituent polymer of the IPN leads to a new system with improved properties, which often are different from those of the individual polymers. Within these IPNs matrix stiffness can be decoupled from scaffold architecture, polymer concentration or adhesion ligand density.

Alginate, a U.S. Food and Drug Administration (FDA)-approved polymer, is one of the most studied building blocks of IPNs because of its versatility and tailorable mechanical properties. Due to its outstanding properties in terms of
biocompatibility, biodegradability, non-antigenicity and chelating ability, alginate has been widely used in a variety of biomedical applications including tissue engineering, drug delivery and in some formulations preventing gastric reflux\textsuperscript{86}. Alginate is a naturally occurring anionic and hydrophilic polysaccharide and is derived primarily from brown seaweed and bacteria. Alginate contains blocks of (1–4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) monomers (Figure 2-12).

Typically, the blocks are composed of three different forms of polymer segments: consecutive G residues, consecutive M residues and alternating MG residues. The state of crosslinking of alginate hydrogels can be controlled by adding or removing divalent cations, with Ca\textsuperscript{+2} being the most commonly used in the form of calcium chloride (CaCl\textsubscript{2})\textsuperscript{87,88}.

Gillette \textit{et al.}\textsuperscript{17} developed a system of IPNs made of type I collagen and alginate to reversibly switch the 3D mechanical microenvironment. IPNs hydrogels were formed by first allowing the type I collagen to gel, followed by the crosslinking of the alginate phase by supplementation of CaCl\textsubscript{2} solution.
Subsequently, the alginate network of the IPNs was uncrosslinked by addition of sodium citrate (a Ca\textsuperscript{2+} chelator). Switching from the crosslinked to the uncrosslinked state not only correlated with a bigger mesh size of the IPN, but also influenced cell morphology and migration. 10T1/2 fibroblasts embedded into these IPNs and subjected to uncrosslinking of the alginate showed an overall increase in cell area (from \( \approx 500\mu\text{m}^2 \) to \( 2000\mu\text{m}^2 \)). Cell spreading and migration were restricted when the alginate component was crosslinked, however switching to the uncrosslinking state led to cell spreading which couldn’t be reverted upon recrosslinking (Figure 2-13).

Figure 2-16 | Dynamic switch of IPNs. Time-lapse bright field images of fibroblasts in collagen-alginate IPNs upon dynamic switching of the alginate network. Scale bar 50 \( \mu\text{m} \).

Another study employing type I collagen and alginate IPNs investigated the behavior of dermal fibroblasts to different stiffness in the context of a model wound dressing biomaterial\textsuperscript{89}. Within this system, the storage modulus could be tuned from 50 to 1200 Pa by solely controlling the extent of crosslinking with calcium. Furthermore the degree of calcium crosslinking didn’t change gel architecture or porosity nor small metabolites diffusion. The cells spread extensively in the soft IPNs, but remained round in hydrogels of higher stiffness, suggesting that it is
possible to modulate the morphology and contractility of fibroblasts infiltrating a wound dressing simply by controlling the storage modulus of the biomaterial itself. More importantly, this study showed that tuning the storage modulus of the IPNs also induced different wound healing-related gene expression profiles. Indeed, a higher gene and protein levels of COX2 and IL-10 was measured in cells cultured in a stiffer IPN. The anti-inflammatory cytokine IL-10 regulates the cytokine network behind inflammation, and is also known to regulate COX-2 during acute inflammatory responses. Inflammation is a key aspect of wound healing, therefore the ability of a wound dressing biomaterial to induce or suppress the expression of key modulators of inflammation is of major importance.

Altogether these studies demonstrated the high versatility of IPNs hydrogels and how their properties can be modulated to regulate cell behavior in different contexts, leading to new strategies for tissue engineering and mechanobiology application.

2.8 Hydrostatic pressure

A considerable body of data exists demonstrating that the application of physiological levels of mechanical stimuli, such as hydrostatic pressure (HP), can influence MSC lineage commitment. For instance, MSC cultured in both osteogenic and chondrogenic media subjected to 1 MPa of cyclic HP (CHP) resulted in a marked increase of proteoglycan as well as an upregulation of mRNA level of chondrogenic genes such as SOX9, ACAN (aggrecan) and COL2 (collagen type II). Similarly, Carroll et al. found that the application of 10 MPa of CHP
on porcine MSC led to the generation of a functional cartilaginous tissue and the suppression of tissue calcification (see Figure 2-17).

**Figure 2-17 | Dynamic HP enhances the functional development of cartilage tissue engineered with bone marrow derived MSC**

**a**

<table>
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**b**

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<tr>
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<tr>
<td>Collagen Type II</td>
<td>BMSCs</td>
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</table>

*Dynamic HP enhances the functional development of cartilage tissue engineered with bone marrow derived MSC*. 

a) Biochemical analyses and b) histological staining of bone marrow derived MSC encapsulated in 4% agarose and cultured in chondrogenic medium for 5 weeks.

The mechanism by which such mechanical stimuli control MSC fate determination is still largely unknown. However, Steward et al. examining the interplay between matrix stiffness and HP in regulating MSC chondrogenesis found that a stiff environment reduced the chondrogenic differentiation of MSC, while enhancing the mechanotransduction to CHP. Inhibiting cellular interaction with the stiff ECM increased cartilage matrix synthesis in free swelling culture conditions, but also suppressed the beneficial effects of CHP. While no difference was found in the morphology of cytoskeletal components such as actin, vinculin, and tubulin,
the authors found that the intermediate filament vimentin was depolymerised in MSC embedded in a stiff ECM when subjected to CHP (see Figure 2-18).

Figure 2-18 | Influence of hydrostatic pressure on the cytoskeletal organisation of MSC. Confocal analysis of MSC stained for vinculin (cyan), actin (red), vimentin (green) and tubulin (magenta) cultured into stiff (4%) hydrogels ± HP. FS: free swelling (control group), HP: hydrostatic pressure (loaded group). Scale bars: 50 μm and 12,5 μm.

Inhibiting cellular interaction with the stiff ECM protected vimentin from depolymerising in response to CHP. Together these results suggest a role for cell-matrix interactions and vimentin in the mechano-transduction of HP.
Another proposed element of HP mechanotransduction is fluctuations in intracellular ion concentration. In particular, calcium ion (Ca\(^{++}\)) signalling has been implicated as a critical regulator of cellular response to several mechanical stimuli\(^{100}\). Steward \textit{et al}\(^{101}\) analysed the behaviour of MSC exposed to HP in the presence of pharmacological inhibitors of Ca\(^{++}\) mobility and downstream signalling molecules. MSC embedded into stiff agarose hydrogels and stimulated with HP significantly increased sGAG and collagen type II deposition, confirming the previous findings. However, supplementation with an intracellular calcium chelator suppressed the beneficial effect of HP. The authors next examined the effect of HP on focal adhesions and the cytoskeleton. Neither the pharmacological inhibitors nor application of HP affected focal adhesion formation. No changes were observed in actin or tubulin structure with either the inhibition of Ca\(^{++}\) mobility or the application of HP, but the vimentin architecture was again more diffusive with HP stimulation. Blocking Ca\(^{++}\) motility abrogated this response to HP. Vimentin retained a more punctate structure in all the treated groups, except when treated with an inhibitor for stretch-activated calcium channel, suggesting that the mechanotransductive pathways utilised in response to cyclic HP are distinct from other loading modalities. Finally, molecules downstream the Ca\(^{++}\) signalling, such as calmodulin, calmodulin kinase type II and calcineurine were inhibited. The use of such drugs abrogated the beneficial effect of HP on sGAG and collagen type II accumulation, with no changes observed in vimentin architecture. This might suggest that Ca\(^{++}\) mobility and associated targets act upstream of changes of vimentin structure. Overall, HP drove the chondrogenic response of MSC by altering Ca\(^{++}\) motility and Ca\(^{++}\) signalling-dependent vimentin reorganization. Although, inhibition of Ca\(^{++}\) signalling may abrogate the chondrogenic
mechanoresponse to HP via a different mechanism to that in which it modulates vimentin structure.
Chapter 3 Materials and Methods

3.1 Cell culture.

Porcine bone marrow-derived mesenchymal stem cells (MSC) were isolated from the femora of 4 months old porcine donors (50 kg) within 2h of sacrifice and preserved in liquid nitrogen before later being thawed and expanded. Tri-potentiality was confirmed prior to use (Figure S1). MSC were plated at a seeding density of 5.000 cells/cm\(^2\) in culture flasks (Nunclon; Nunc, VWR, Dublin, Ireland) maintained in growth medium (GM) composed of high-glucose Dulbecco’s modified eagles medium (4.5mg/mL D-Glucose, 200mM L-Glutamine; hgDMEM) supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/mL)–streptomycin (100 mg/mL) (all GIBCO, Invitrogen, Dublin, Ireland) and expanded to passage 3 in a humidified atmosphere at 37 °C and 5% CO\(_2\). For differentiation studies, MSC were supplemented with chondrogenic differentiation media (CDM) composed of hgDMEM, penicillin (100 U/mL) and streptomycin (100 mg/mL), 100 mg/mL sodium pyruvate, 40 mg/mL L–proline, 1.5 mg/mL bovine serum albumin, 4.7 mg/mL linoleic acid, 10 insulin-transferrin-selenium, 50 mg/mL L–ascorbic acid–2– phosphate (all Sigma–Aldrich, Arklow, Ireland), 100 nM dexamethasone (Sigma–Aldrich) and 10 ng/mL TGF–β3 (R&D Systems, Abingdon, UK). Cells were cultured in DM for 7 days at 5% oxygen, in a humidified atmosphere at 37 °C and 5% CO\(_2\).
3.2 Mechanical testing.

 Constructs were mechanically tested \((n \geq 5\) each group) in unconfined compression between impermeable platens using a standard materials testing machine with a 5 N load cell (Zwick Roell Z005, Herefordshire, UK) hydrated through immersion in a DMEM bath maintained at room temperature. A preload of 0.01 N was applied to ensure that the construct surface was in direct contact with the impermeable loading platens. Ramp tests were performed consisting of a ramp displacement of 1 µm/s up to 30% strain. The initial elastic modulus was measured as the slope of the stress–strain curve between 5 and 15% strain.

3.3 3D IPN samples histology.

 For immunological staining, the samples were fixed for 1 hour in 4% PFA for alginate at +4°C in rotation. Gels were then rinsed in PBS and incubated overnight in 30% sucrose in distilled water at +4°C. The samples were then placed in a mix of 50% of a 30% sucrose in distilled water solution, and 50% OCT (Tissue-Tek) for 5 hours at +4°C. Finally, the samples were placed in OCT and frozen in a isopentane (Merck) bath previously chilled in liquid nitrogen and stored at -80°C. Sections of 40 µm were cut with a cryostat (Leyca CM 1860) and mounted on glass slides (VWR) previously coated with gelatin, left to dry for 30 minutes and stored at -20°C until stained.

3.4 Immunostaining.

3.4.1 2D IPN sample preparation

 Samples were fixed in 4% paraformaldehyde for 20 minutes, washed in PBS and stored at +4 °C until imaged. For the immunofluorescence analysis, samples were
permeabilised in 0.5% Triton-X-100 (Merck), rinsed thrice in PBS and incubated in blocking buffer containing 5% bovine serum albumin (BSA, Merck) before adding the corresponding primary and secondary antibodies. See Table 1 for the complete list of antibodies and reagents. Samples were mounted with Vectashield antifade (Vector Laboratories) and placed on an imaging dish (µ-Dish 35mm, iBidi).

3.4.2 3D IPN sample preparation

Cryoslices (at least 3 slices per sample, n = 3) were left to re-equilibrate at room temperature for 5 minutes while single slices boundaries were drawn with a pappen (Ted Pella). Samples were permeabilised in 0.5% Tritont-X-100 (Merck), rinsed once in PBS and incubated in blocking buffer containing 5% bovine serum albumin (BSA, Merck) before the incubation with the primary and secondary antibodies (Table 1 for complete list of reagents). Samples were mounted with 2.5 μl of ProLong gold antifade (Thermo Fisher scientific) on each slice of sample, covered with a coverslip (VWR) and left to cure for 1 hour at room temperature and then overnight at +4°C.
Table 1 | List of specific antibodies and toxins used

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<td>Phalloidin-iFluor 647 - cytopainter</td>
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<td>DAPI</td>
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3.5 Image quantification

Samples were imaged the next day with a Leica SP8 scanning confocal microscope (equipped with lasers for 405nm, 488nm, 552nm and 638nm and 3PMT detectors) with X 20 or X 40 (1.3 numerical aperture, oil-immersion) objective lens. Z-stack images were acquired with an interval of 1 μm, using the same exposure, gain and offset values for all conditions in the same experiment. These parameters were set based on positive controls expressing the protein of interest, and negative controls obtained by omitting the primary antibody. YAP, SMAD 2/3 and HDAC4 nuclear quantification and NCAD total quantification were calculated using a custom-made code for FIJI 3D Image Suite for the 3D mask generation, followed by the formula:

\[ \frac{\sum_{nuc}^f}{V_{nuc}} \quad \text{or} \quad \frac{\sum_{cyto}^f}{V_{cyto}} \]
Where $\sum_{\text{nuc}}^f$ and $\sum_{\text{cyto}}^f$ represent the sum of the background-corrected intensity values for the voxels in the nuclear and cytoplasmic region respectively, while $V_{\text{nuc}}$ and $V_{\text{cyto}}$ the volume of the corresponding regions.

### 3.5.1 Specific image quantification method for 2D IPN

Focal adhesion (FA) quantification was performed with FIJI adjusting the previously reported method by Horzum et al.\textsuperscript{102,103}. To quantify colony number and area, a scan of the entire surface of the 2D IPN substrate ($n \geq 3$ per group) was taken and reconstituted with the mosaic function of LAS X confocal software (Leica). The actin signal thresholded and the ROI detected with the Analyse Particle function of FIJI. Using the corresponding DAPI channel as reference, the colonies were distinguished from single cells based on the average area dimension of the respective ROI and the presence of cell-cell contact. Single cell circularity was analysed using the Measure function in FIJI.

### 3.5.2 Specific image quantification method for 3D IPN

To quantify COL2 positive cells, the masks of the cells obtained from the thresholded actin staining, were superimposed on the COL2 binarized channel, cell-masks matching with COL2 signal were considered positive for its expression. To quantify the cell colonies, the actin signal was thresholded and the ROI detected with the 3D manager function of FIJI. Using the corresponding DAPI channel as reference, the colonies were distinguished from single cells based on the average volume dimension of the respective ROI and the presence of cell-cell contact. Single cell sphericity and volume were analysed using the Measure function in the 3D Image suit of FIJI. Cell-Cell proximity in 3D was calculated using the ROI
derived from the actin signal, the minimum border-to-border distance was automatically computed using the Distance function of FIJI’s 3D Manager.

### 3.6 FIJI macro code for image processing.

A custom-made macro code for FIJI\(^{104}\) software was developed to streamline the quantification process of 3D images obtained from confocal microscopy (see annex II to review the full code). The code was designed to analyse an image at a time by firstly, generate and highlight 3D masks of the regions of interest (ROI) (e.g. cell nucleus, cytosol) within a given z-stack image and secondly, to superimpose those masks on the pre-processed image to quantify the signal intensity for the channel of interest. A list of ROI will be generated and saved in a zip file. The code begins by acknowledging the image title and path, the latter used as location to store the zip file for the ROI (Figure 3-1, line 1 to 5). Next, a sequence of arrays is available to (Figure 3-1):

1. name the channels present in the image, line 8
2. indicate the channels to be analysed, line 9
3. select the first thresholding algorithm to generate the masks, line 10
4. select the second thresholding algorithm for the image quantification, line 11
5. define the minimal pixel size that describes the volume of the region of interest when inscribed in a bounding box, line 12
Figure 3-1 | Macro code from line 1 to line 13. Macro opened with FIJI script editor. It is possible to modify the parameters written in pink or purple to customize the image processing.

```java
1 imageTitle = getTitle();
2 imageRoot = File.directory;
3 print(imageRoot);
4 path_for_results = imageRoot;
5 Channels = newArray("DAPI", "COL2", "SMAO23", "F-ACTIN");
6 ActiveChannels = newArray("ON", "OFF", "ON", "ON");
7 AutoThresholdMethod1 = newArray("Otsu", "Otsu", "Otsu", "Otsu");
8 AutoThresholdMethod2 = newArray("Otsu", "Otsu", "Otsu", "Otsu");
9 VolBoundingLimit = newArray(1000, 1000, 2000, 2000);
```

A window will pop up at the beginning of the analysis to remind to check and confirm the parameters and the destination folder for the zip files. It is possible to remove this safety feature by simply deleting line 15 of the code. Next, the channels of the image will be split and treated one at a time (only the active channels selected in Figure 3-1, line 9). Flexible segmentation workflows are user-defined. They can vary depending on the specific datasets to be processed, generally based on the positive and negative controls for the given immunofluorescence and kept constant among all the conditions. The pre-processing of the image via selection of 3D Gaussian Blur filter is necessary to facilitate subsequent thresholding (Figure 3-1, line 10) and watershed algorithm application when necessary. The binary image obtained is then segmented with the 3D Manager, a built-in plugin of FIJI. The Add Image command present in the 3D Manager will generate a list of ROI. At this point, only the ROI with a size bigger than the one set in line 12 (Figure 3-1) will be selected and saved in a zip file named as the channel of origin. Finally, when all the channels have been processed, the 3D Manager and the original image will be re-opened, the latter split in the channels to be analysed, thresholded (thresholding
limits set in Figure 3-1, line 11) and resized to fit in the field of view of the screen. The user is now able to manually open the zip file with the 3D Manager and for instance, superimpose the ROI of the nuclei on a second channel labelling a protein of interest and quantify the fluorescence intensity within the nuclei masks in 3D.

### 3.7 Live/Dead staining of 3D IPN

Cellular viability was investigated before the media change to CDM by using a live/dead assay solution consisting of Calcein-AM (1 mM) and Propidium Iodide (0.1 mM) prepared in phenol-red free DMEM (4.5mg/mL D-Glucose, 200mM L-Glutamine; hgDMEM, GIBCO). Briefly, samples were rinsed in PBS, immersed in the staining solution for 1 hour at 37°C. Then, samples were rinsed twice with phenol red free DMEM and left in warm phenol red free DMEM before imaging. Live imaging was performed using a confocal microscope (Leica Confocal Microscopy TCS SP8) using 490/ 515 nm (excitation/emission) for Calcein-AM (Life Technologies) and 535/617 nm (excitation/emission) for propidium iodide. Maximum intensity projection images were obtained using FIJI software.

### 3.8 RNA isolation from MSC cultured on top of 2D IPN.

Total RNA was extracted from MSC seeded 2D IPN after 7 days of culture. Cells were harvested using a cell scraper and lysed in 1 ml of TRI Reagen (Merck) followed by chloroform extraction as per manufacturer’s instructions. RNA was re-suspended in RN-ase free water and its concentration determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fischer).
3.9 RNA isolation from MSC embedded into 3D IPN

The gels (n = 5 per group) were washed once in PBS, mechanically digested and then incubated with 1 ml of alginate dissolving buffer (0.055 M sodium citrate, in 0.03 EDTA, 0.15 M NaCl, pH 6.8 (all Merck)) for 10 minutes at 37 °C. Samples were then centrifuged for 2 minutes at 10,000 rpm, rinsed in PBS and centrifuged for 2 minutes at 14,500 rpm. The resulting pellets were lysed with 350 μl of RLT buffer containing 1% β-Mercaptoethanol with the latter addition of 540 μl of RNA-free water and 10 μl of Protease K solution (Qiagen) and kept for 10 minutes at 55°C. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) following manufacturer instructions, snap frozen in liquid nitrogen and stored at -80°C.

3.10 Gene expression analysis

Polymerase chain reaction (PCR) with a High capacity cDNA reverse transcription kit (Thermofisher) was conducted to transcribe 300ng of RNA from each sample into cDNA. After cDNA quantification with Qubit ssDNA Assay kit (Thermofisher), levels of gene expression was measured with real-time PCR (ABI 7500-fast, Applied Biosystems) using SYBR green master mix (Applied Biosystems) and porcine specific primers (Table 2). The relative quantity of each sample was calculated with the Pfaffl105 method with reference to 18S and B2M and expressed as fold change to the control group (specified in each figure legend). Efficiency of all primer pairs were calculated by serial dilutions of RNA isolated from porcine skeletal muscle tissue (YAP and CYR61) or day 7 MSC pellets (for all other genes).
Table 2 | List of specific primers for real time PCR.

<table>
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<tr>
<th>Gene name</th>
<th>Gene full name</th>
<th>Forward/Reverse</th>
<th>T(°C) in use</th>
<th>T(°C) predicted</th>
<th>Gene ID</th>
<th>Gen Bank No.</th>
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<td>B2M</td>
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<td>F:5’ ACTGAGTTCCATCTCTCTAAACG 3’</td>
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<td></td>
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<td>R:5’ TGCAGCATCTGCATAACCTTC 3’</td>
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<td>JBP</td>
<td>ribosomal protein 518</td>
<td>F:5’ CAACACCACATGAGCATATC 3’</td>
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<td>59</td>
<td>396980</td>
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<td>aggrecan</td>
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<td>397255</td>
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</tr>
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<td></td>
<td>57.1</td>
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</table>

### 3.11 Statistical analysis.

All experiments were conducted using at least three replicate samples per condition.

Statistical analyses were performed using one- or two-way analysis of variance (ANOVA) with a post hoc Tuckey’s multiple comparison test used to make pairwise comparisons between multiple groups or a two-tailed Student’s t-test followed by a Mann Whitney test when only two groups were being compared using GraphPad Prism 6.0 statistical software (GraphPad Software). Significance was accepted at a level of p < 0.05. Numerical and graphical results are presented as mean ± s.e.m..
Chapter 4  Substrate stiffness and cell-cell interactions regulate chondrogenesis of mesenchymal stem cells

4.1  Introduction

Cartilage injuries and poor capacity to self-repair often lead to osteoarthritis (OA), which is estimated to affect more than 40 million people across Europe\textsuperscript{106}. OA is the most frequent chronic joint disease and a major contributor to functional disability and loss of autonomy in older adults\textsuperscript{107}. Current cell based therapies for cartilage defect repair such as autologous chondrocyte implantation suffer from significant drawbacks, such as donor site morbidity and dedifferentiation of chondrocytes during monolayer expansion. Mesenchymal stem cells/marrow stromal cells (MSC) are an attractive alternative cell source for cartilage tissue engineering due to their clinical relevance and multipotency\textsuperscript{3}, but their safe and efficacious use requires the development of strategies to control their differentiation into specific cell types. It is well established that cell-cell\textsuperscript{108,109} and cell-matrix interactions, and in particular the mechanical properties of the cellular microenvironment\textsuperscript{5,8,12,25,53,110–112}, can direct MSC differentiation. Interpretation of extracellular matrix (ECM) stiffness requires the engagement of focal adhesions (FA) through which the cell actively interrogates its surroundings\textsuperscript{113}. Designing biomaterials that match the stiffness of specific tissues is emerging as a promising tissue engineering strategy to control MSC fate\textsuperscript{10,114}. For instance, a compliant substrate (1 kPa) is able to guide MSC towards a neural lineage, a stiffer substrate
(10 kPa) will induce the formation of myotubes, while a rigid substrate (40 kPa) will support the development of osteoblasts\textsuperscript{5,25,53}. Although the role of substrate stiffness on MSC differentiation has been investigated, the majority of studies have focused on the role of such mechanical cues in regulating osteogenesis and adipogenesis of MSC, with less focus on the role of matrix stiffness in regulating the initiation and progression of chondrogenesis.

Chondrogenesis is typically induced \textit{in vitro} by incubation of MSC in a defined media containing TGF-\(\beta\) in a three-dimensional (3D) pellet culture\textsuperscript{2}. During limb bud development, mesenchymal precursors condensate forming cell-cell interactions mediated by N-Cadherin (NCAD)\textsuperscript{5,114} and secrete the growth factor TGF-\(\beta\). TGF-\(\beta\) regulates chondrogenesis by activation of SMAD 2/3 transcription factors, which lead to the stabilisation of the SOX9 transcription complex\textsuperscript{59,61}. NCAD is a cell surface protein, and deletion of the extracellular or intracellular domains, or blocking NCAD interactions via antibody treatment, inhibits condensation and subsequent chondrogenesis\textsuperscript{5,8,12,21,69,115,116}. Recent studies also point to a role for substrate stiffness in directing the initiation and progression of chondrogenesis. Soft substrates (~1 kPa) have been found to favour MSC commitment towards chondrogenesis when combined with the supplementation of TGF-\(\beta\)\textsuperscript{69}. Other studies have reported that matrix stiffness in the range of articular cartilage (0.5 MPa) better support a chondrogenic phenotype, which was associated with a matrix stiffness-sensitive induction of TGF-\(\beta\)\textsuperscript{68}.

Exploring the role of matrix stiffness in regulating chondrogenesis of MSC required the development of biomaterials with user defined stiffness. Different materials have been developed to analyse the role of ECM mechanics in regulating MSC behaviour, from differentiation\textsuperscript{8} to migration\textsuperscript{117}. However, decoupling the effect of matrix stiffness from other factors such as ligand density or matrix
composition can be challenging. One way to overcome these limitations is the use of interpenetrating network (IPN) hydrogels, which are made of at least two polymer networks that are partially interlaced\textsuperscript{89,118}. Interpenetrating networks of type I collagen and alginate have recently been proposed as model systems to explore how matrix stiffness regulates cell fate\textsuperscript{17,119}. Type I collagen provides cell binding domains and it is representative of many \textit{in vivo} contexts, while alginate, which is a polysaccharide from seaweed known for its biocompatibility and abundance\textsuperscript{86}, is used to tailor hydrogel mechanical properties by varying its concentration or crosslinking. MSC seeded on top of (or within) these IPN substrates bind and pull on the collagen-I fibrils, and depending on the overall rigidity of the IPN, they will experience a defined resistance to deformation from the matrix.

The objective of this study was to examine the interplay between matrix stiffness and cell-cell interactions in regulating chondrogenesis of MSC. We hypothesise that a combination of a soft substrate and a high cell density will enhance the initiation of MSC chondrogenesis. To test this hypothesis, marrow derived MSC were seeded at low or high densities on the surface of IPN hydrogels of differing stiffness, and maintained in chondrogenic media containing TGF-β3 in a physiologic environment. We then compared cell morphology, SMAD 2/3 and NCAD staining, proliferation levels and gene expression. Our results show that MSC seeded at high density on soft IPN substrates migrate, proliferate and condensate, forming large cellular aggregates expressing NCAD. These findings highlight alternative mechanisms by which MSC will respond to alterations in substrate stiffness while undergoing chondrogenesis.
4.2 Materials and Methods

4.2.1 IPN fabrication and cell seeding.

A volume of 1 mL of 6% ice-cold collagen type I solution (from rat tail, Corning) had the latter addition of 400 µl of 10X RPMI (Merck), 400 µl of collagen neutralization buffer containing 0.1M HEPES and 1M sodium bicarbonate dissolved in PBS and 350 µl of GM for volume adjustment. The resulting collagen solution was thoroughly mixed with 2 ml of 1% or 3.5% alginate solution (LVG UP, batch# BP 0907-02, viscosity = 198 mPa*s, Pronova matrix) to have soft or stiff IPN, respectively. Then, 1ml of biomaterial have been pipetted in between two slabs of 3% agarose (Merck A9539) containing 100mM CaCl₂ and stored in a well of a 6-well plate (Figure 4-1a), covered with DMEM and incubated overnight in a humidified atmosphere at 37 °C and 5% CO₂. After the incubation time, the IPN substrates were removed from the agarose slabs, rinsed with DMEM and stored in incubation in GM until cell seeding. After removal of GM, MSC were seeded at a density of 15000 cell/cm² (for high density studies) or 3000 cell/cm² (for low density studies) and flushed with fresh GM after 1 hour from the seeding time. After additional 12 hours, GM was replaced with starvation media composed of hgDMEM, penicillin (100 U/ mL) and streptomycin (100 mg/mL) and 0.5% FBS for 24 hours, to allow cell cycle synchronization, later replaced by CDM. Samples were cultured for 7 days at 5% oxygen, in a humidified atmosphere at 37 °C and 5% CO₂.

4.2.2 DNA biochemical analysis

Porcine MSC were seeded onto IPN substrates (n ≥ 3 per group), maintained for 12 hours in growth medium, 24 hours in starvation medium followed by DM.
Immediately before the DM treatment and after 7 days, cells were rinsed twice with cold PBS and lysed on ice for 20 minutes with 100 μL lysis buffer containing 0.2% v/v Triton X-100, 10 mM Tris pH 8 and 1 mM EDTA (all Merck). Cells were detached using a cell scraper, vortexed thoroughly and stored at −80 °C. The cell suspensions were homogenised with a 21G needle before measuring DNA concentration using Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermoscientific) following the manufacturer protocol.

4.2.3 Metabolic activity

The metabolic activity of treated cells was evaluated using a standard Alamar Blue (AB) assay (Biosciences, Ireland). For the assay, cell culture media from the culture wells was replaced with 10% AB in phenol-red free media (1 mL/ well) and incubated for 4h at 37 °C. As negative control, 10% AB solution was added to empty wells. After the incubation time, 200 μl of the supernatant was plated in triplicate into a 96-well plate; absorbance was read at 570 and 600 nm using a standard spectrophotometer (Bioteck, Instruments Inc, UK). The values of AB optical density (O.D.) were corrected for background values of negative controls and showed as % of AB reduction following the manufacturer’s protocol.
4.3 Results

4.3.1 IPN stiffness modulates YAP localization

An IPN hydrogel of alginate and type I collagen was developed to assess the influence of matrix stiffness on the chondrogenic differentiation of MSC (Figure 4-1a). The stiffness of this IPN could be increased from 2.8±0.7 kPa (soft) to 18±4.1 kPa (stiff) by increasing the concentration of alginate within the IPN (Figure 4-1b). To validate this biomaterial system as a tool for exploring cellular mechanobiology, MSC were cultured on the surface of these hydrogels in growth medium as previously established\textsuperscript{24}. After 3 days in culture, MSC were able to generate actin stress fibres and to assume a reasonably spread morphology on both soft and stiff substrates (Figure 4-1c). In such conditions, YAP localization was found to be mainly cytosolic in MSC grown on the surface of soft IPN, nucleo-cytosolic in MSC atop of stiff IPN, and mainly nuclear when seeded onto tissue culture plastic (TCP) (Figure 4-1d).
Figure 4-1 | Mechanical characterization of IPN substrates, morphological evaluation of MSC and YAP localization. a) Schematic of IPN fabrication. b) Young’s Modulus of soft and stiff IPN immediately after fabrication, **P<0.01. c) Confocal analysis of MSC seeded on soft and stiff IPN substrates. Cells were stained for actin (red) and nuclei (blue). Scale bars, 10 μm. Graph: quantification of actin circularity of single MSC cultured onto IPN substrates. d) Confocal
images of MSC cultured onto surfaces of different stiffness: soft IPN, stiff IPN and tissue culture plastic (TCP). Cells were stained for actin (red), YAP (green) and nuclei (blue). Scale bar, 10 μm.

4.3.2 Changes in MSC shape and focal adhesion assembly in response to alterations in substrate stiffness depend on cell density

Higher cell densities typically support the chondrogenic differentiation of MSC\textsuperscript{103,120}, however such cell crowding can override the influence of substrate stiffness\textsuperscript{121}. To better understand the influence of cell seeding density on the response of MSC to different substrate stiffnesses, we examined cellular shape and focal adhesion (FA) assembly on soft and stiff substrates seeded at low (3,000 cells/cm\textsuperscript{2}) and high (15,000 cells/cm\textsuperscript{2}) cell densities. After 3 days in chondrogenic differentiation media (CDM), MSC seeded at a low density assumed a spread morphology and assembled a similar number of FA per cell on soft and stiff substrates (Figure 4-2a). At higher cell seeding densities, MSC assembled a larger number of FA per cell when cultured on stiff substrates, and adopted a more spread morphology compared to cells cultured on a softer substrate (Figure 4-2b).
Figure 4-2 | Effect of cell seeding density on FA assembly. Confocal analysis of MSC seeded at a) low or b) high density onto soft and stiff IPN substrates. Cells were stained for actin (red), vinculin (green) and nuclei (blue). Scale bars,
10 μm. Graphs: quantification of total number of FA per cell and the morphological descriptor of actin circularity, **p<0.01, ****p<0.0001.
4.3.3 Soft substrates support cell condensation and SMAD 2/3 nuclear entry

Having demonstrated that MSC adhere to the surface of the IPN, and that during chondrogenesis MSC adopt different shapes depending on the substrate stiffness and local cell density, this thesis next sought to determine if changes in FA number and cell morphology were associated with altered cellular signaling associated with chondrogenesis. To this end, the expression and localization of NCAD (a marker of cell condensation\(^{122}\)), SMAD 2/3 transcription factors (involved in the initiation of chondrogenic differentiation \(^{123}\)), and YAP (which has been associated with both promoting and suppressing chondrogenesis\(^{82,83}\)) were analysed in MSC seeded onto soft and stiff substrates. At both cell seeding densities, NCAD signal was higher when MSC were seeded on soft IPN, (Figure 4-3a and b). As expected, NCAD was preferentially localised around the periphery of cells.
**Figure 4-3 | MSC condensation on IPN.** Confocal images of NCAD distribution in MSC seeded at a) low and b) high density. Cells stained for NCAD (green), actin (red) and nuclei (blue). Scale bar, 10 µm. Graphs: quantification of NCAD expression level, *p<0.05.

SMAD 2/3 was found to be nucleo-cytosolic in MSC growing on soft substrates, while mainly confined to the cytosolic compartment in MSC on stiff substrates (Figure 4-4a and b). This was observed for both the low and high seeding density. In contrast, YAP localisation depended on the local seeding density. At low seeding densities, YAP preferentially localized in the cytosol on soft substrates, but was more nucleo-cytosolic in MSC on stiff substrates (Figure 4-4c). In contrast, at high seeding densities the increased cell-cell interactions correlated with increased nuclear YAP on soft substrates, while it remained confined to the cytosol in MSC on stiff substrates (Figure 4-4d).
Figure 4-4 | Soft IPN drive SMAD 2/3 and YAP nucleo-cytosolic translocalization when MSC are seeded at high density. Confocal analysis of SMAD 2/3 and YAP distribution in MSC seeded at a&c) low and b&d) high density respectively. Cells stained for SMAD 2/3 (magenta) or YAP (green) and nuclei (blue). Scale bar, 5 µm in a,b & c and 10µm in d. Graphs: quantification
of SMAD 2/3 and YAP expression level in the nuclear compartment graphed as fold change of the soft group, *p<0.05, ****p<0.0001.
4.3.4 MSC form larger aggregates on soft substrates and undergo more robust chondrogenesis

To further characterize the chondrogenic differentiation of MSC, the degree of cell proliferation and cellular aggregation as a function of substrate stiffness and seeding density was next assessed. A ~3 fold increase in total DNA levels (indicative of proliferation) was observed for cells seeded at the higher seeding density by the end of the culture period (Figure 4-5a), with no significant change in DNA levels observed for the low density group. The stiffness of the IPN did not significantly affect the proliferation of MSC, although a trend towards a higher number of Ki67+ cells (Figure 4-5b) and increased metabolic activity (Figure 4-5c) was observed in MSC cultured on stiff substrates. While limited aggregation was observed at low density (data not shown), at the high density MSC were observed to form a similar number of aggregates per mm² (Figure 4-5e), however the size of the aggregates was significantly larger when grown on soft substrates (Figure 4-5e).
Figure 4-5 | Soft IPN enhance cell condensation. a) DNA content of samples seeded at low or high density at day 1 and day 7, normalized to the area of the IPN, ** p<0.01. b) Quantification and localization of Ki67+ cells in day 7 high density seeded IPN. Cells stained for Ki67 (green), actin (red) and nuclei (blue), scale bar 10 µm. c) Metabolic analysis of day 7 high density seeded samples. d) Localization of cell clusters in soft or stiff IPN seeded at high density and their respective colour-coded area (px²). Cells stained for actin (magenta), scale bar 1mm. e) Quantification of aggregates number per mm² and average size of aggregates, *p<0.05.
At lower seeding densities, substrate stiffness had no significant effect on the expression of the chondrogenic marker genes SOX9, ACAN and COMP (Figure 4-6a). COL2 did not amplify at the lower seeding density (data not shown). The expression of TGFBR2 (TGFβ receptor II) and CDH2 (N-cadherin) was higher in MSC grown on the soft IPN.

Figure 4-6 | MSC cultured at high density on soft IPN chondrogenically differentiate. MSC gene expression relative to soft group after 7 days of culture at a) low or b) high density. *p<0.05, **p<0.01, ***p<0.001.
Increasing the initial cell seeding density enhanced the expression of chondrogenic markers on both soft and stiff substrates (Figure 4-7).

**Figure 4-7 | Higher cell density enhances the chondrogenic response of MSC.**

Effect of seeding density on MSC gene regulation after 7 days of culture on a) soft or b) stiff 2D IPN, *p<0.05, **p<0.01, ***p<0.001.

At higher seeding densities, the expression of *SOX9, ACAN, COL2, TGFBR2* and *CDH2* was higher on soft substrates (Error! Reference source not found.b). For both low and high seeding densities, *YAP* expression was not affected by the stiffness of the substrate, however *CYR61* expression (suggestive of higher YAP transcriptional activity) was higher on soft substrates at low densities, and higher on stiff substrates at higher cell densities.
4.4 Discussion

The objective of this study was to investigate the role of both substrate stiffness and cell density on the chondrogenic differentiation of MSC. To enable this investigation, an IPN consisting of type I collagen and alginate was developed, where the stiffness of the hydrogel was tuned by altering the concentration of alginate within the IPN. While this study explored the differentiation of MSC seeded onto the surface of such hydrogels (a 2D culture), the advantage of this system is that it also supports the growth of cells encapsulated within the hydrogel (i.e. a 3D culture). This will be done in subsequent chapters of this thesis. In this chapter we found that MSC seeded onto the surface of hydrogels (i.e. 2D culture) undergo more robust chondrogenesis when maintained at high densities. In such an environment, MSC form larger aggregates on soft substrates, which correlated with increased cellular circularity, lower levels of FA assembly, increased NCAD expression and nuclear SMAD 2/3 localization and increases in the expression of a number of key chondrogenic genes (Figure 4-8).
MSC were found to adhere and spread on the surface of both the soft and stiff IPN. When maintained in growth media (i.e. in the absence of any specific differentiation factors), no substrate stiffness dependent differences in cell morphology were observed, although nuclear YAP localization increased on the stiffer substrates. This is in agreement with earlier studies that observed increased nuclear YAP in MSC maintained on stiff substrates\textsuperscript{24,124–126}. This suggests that MSC are capable of responding to the changing stiffness of the IPN hydrogels in a similar manner to that observed on different materials such as collagen coated polyacrylamide hydrogels.

When MSC were cultured at high density (15000 cells/cm\textsuperscript{2}) in the presence of chondrogenic growth factors, they were able to engage FA in a stiffness dependent manner. The lower number of FA assembled by MSC on the soft substrates correlated with a reduction in cell spreading when compared to MSC on stiffer substrates. A similar trend was also observed for MSC seeded at low density, although they typically assumed a more spread morphology irrespective of substrate stiffness in these conditions, perhaps in the attempt to sense and interact

**Figure 4.8 | High cell density and soft substrates support MSC chondrogenesis.**
with neighbouring cells. FA act as a dynamic interface between the cytoskeleton and the ECM, transmitting mechanical forces across the cell membrane\textsuperscript{127,128}. During cell migration, FA connect to the ECM and generate the forces necessary to pull the cell body forward. Subsequently, cells must release from the ECM to continue cell movement. As such, directional migration of the cell requires continuous, coordinated formation and turnover of FA at the leading edge of the cell body and release of this attachment at the rear\textsuperscript{129–131}. Therefore, a cell with a larger number of cell–matrix adhesions would result in a slower migration speed\textsuperscript{132,133}. Such dynamic of cell motility suggests that our soft IPN could enhance the migration of MSC, which were then able to condensate and express higher levels of NCAD than MSC growing on stiff IPN. NCAD is essential for the onset of MSC chondrogenesis, mediating cell-cell contact and condensation\textsuperscript{120,134}.

The nuclear localization of SMAD 2/3 was higher in MSC maintained on the surface of soft IPN, irrespective of the local cellular density. SMAD proteins are a family of signal transduction protein that can be activated by TGF-β. The binding of TGF-β to its receptor TGF-β receptor II (TGFBR2), leads to the transphosphorylation of TGF-β receptor I (TGFBR1), which in association with SMAD 2 activates SMAD 3. At this point, SMAD 2/3 complex dissociates from TGFBR1, develops affinity for SMAD 4 and migrates to the nucleus to regulate the transcription of target genes (i.e. $SOX9$)\textsuperscript{135–138}. In other cell types, YAP and TAZ have been shown to be mechanosensory proteins that bind to SMADs\textsuperscript{77,139–143}. For example, fibroblast growth on a soft matrix promoted YAP/TAZ sequestration in the cytosol and impaired TGF-β–induced SMAD 2/3 nuclear accumulation and transcriptional activity\textsuperscript{141}. Surprisingly, at a higher cell density, higher levels of nuclear YAP were observed in MSC on soft IPN, although in agreement with previous studies, this correlated with higher levels of nuclear SMAD 2/3. In
contrast, at lower seeding densities that did not support robust chondrogenesis, YAP nuclear localization was greater on stiff substrates, however this did not correlate with increased SMAD 2/3 nuclear localization. It is known that YAP activity can be altered by numerous factors such as substrate elasticity, cell density\(^{144}\) and morphology\(^{24,124,125}\) and it is cell type-and context dependent, however its role in regulating chondrogenesis is still unclear\(^{82,83,145,146}\). In vitro YAP resulted downregulated during chondrogenesis of human MSC and its overexpression caused the inhibition of the chondrogenic differentiation of murine C3H10T1/2 cells\(^{82}\). In another report, YAP was found to promote early chondrocytes proliferation and to inhibit subsequent maturation in vitro and in vivo thus, bone formation and repair were strongly impaired\(^{83}\). While YAP contribution seems more clear during MSC osteogenic differentiation\(^{147,148}\) and bone homeostasis\(^{149}\), the same cannot be applied yet for chondrocyte differentiation, since YAP regulates both proliferation and hypertrophy simultaneously. In this study we found that in conditions supportive of chondrogenesis (high cell density, low substrate stiffness), increased nuclear YAP was observed early in the differentiation process, which likely plays a role in regulating SMADs activity. Our findings at lower seeding densities suggest that increased nuclear YAP is not sufficient to promote SMAD 2/3 nuclear accumulation in MSC stimulated with TGF-β3.

MSC proliferation also depended on the initial seeding density. In agreement to what is reported in literature\(^{150-153}\), higher initial seeding densities supported higher levels of proliferation. Substrate stiffness did not appear to influence MSC proliferation. As well as proliferating, MSC were also observed to form aggregates on both soft and stiff substrates, with larger aggregates forming on the softer substrates. A potential explanation for this is higher speeds of cell migration on the softer substrates, as it has been observed also on soft (3kPa) PDMS
substrates\textsuperscript{154}, although this was not explicitly measured. These larger cellular aggregates/colonies correlated with increased expression of key chondrogenic genes. During limb bud development, mesenchymal cells condensate and differentiate into chondroprogenitor cells. The onset of chondrogenesis is defined by the upregulation of the gene \textit{SOX9}, which is in turn responsible for maintaining proliferation\textsuperscript{155}, and directly regulates the expression of the gene \textit{COL2}\textsuperscript{156} resulting in the production of an ECM containing the proteoglycan aggrecan, a marker of chondrocyte differentiation\textsuperscript{157}. Chondrogenesis of MSC is induced \textit{in vitro} by incubation of cells in defined media containing TGF-\textbeta\ in combination with a 3D culture system, typically a micromass pellets\textsuperscript{2,158,159} or cellular encapsulation into hydrogels\textsuperscript{71,160,161}. TGF-\textbeta is the most prominent growth factor controlling MSC chondrogenesis and signals via the receptor-activated SMAD 2 and 3.

A possible explanation to our results can be found in the ability of YAP to control FA assembly\textsuperscript{126} and maturation to enable a persistent cell motility\textsuperscript{162}. A continued transcription/translation is required for continuous cell migration, to replace new FA at the cell leading edge\textsuperscript{162}. As such, we can argue that the nuclear abundance of YAP seen in high density MSC on soft 2D IPN was due to the need of a rapid turnover of the FA structures rather than the overall number of FA at a given time-point. In fact, although a minor quantity of FA could have enhanced MSC migration, a rapid turnover of the same might have been needed to sustain a constant migration, thus explaining YAP nuclear abundance. Moreover, YAP displays a specific DNA-binding signature for genes encoding for proteins involved in cytoskeleton stabilization and connection to the membrane, one among all \textit{CDH2}\textsuperscript{126}, which was upregulated in our system. This evidence reinforces the indication of YAP as a possible target for the chondrogenic differentiation of MSC, however solving this puzzle deserves further investigations.
Although we identified a possible mechanism through which MSC respond to the elasticity of the substrate under chondrogenic conditions, we partially explored how MSC experienced the range of stiffness of the IPN. Our data showed a differential assembly of FA accordingly to the varying stiffness of the 2D IPN and although being outside the aim of our study, an investigation of the specific composition of integrin subunits engaged in the FA could give more insights on MSC environmental patrolling under chondrogenic stimuli. Further, a 2D environment doesn’t exhaustively represent the complex physiological environment of a native tissue, for this reason the remaining chapters of this thesis will explore chondrogenic differentiation of MSC in a 3D contest, exploiting the versatility of our IPN.

In conclusion, we have shown that MSC chondrogenic differentiation in response to TGF-β3 stimulation is dependent upon the substrate mechanical properties and cell density. More importantly, this study demonstrates that biomaterials mimicking in vitro the stiffness of the developing cartilage tissue\textsuperscript{163,164} support chondrogenesis of MSC, at least in part by supporting cellular condensation. The data presented in this study highlight the importance of considering how MSC respond to extrinsic mechanical stimuli in order to develop biomaterials strategies able to tailor MSC fate determination in the context of stem cell based therapies for cartilage tissue regeneration.
Chapter 5  The role of cell shape and hydrogel stiffness in determining chondrogenesis of mesenchymal stem cells.

5.1  Introduction

The inability of adult articular cartilage to regenerate even relatively small defects has motivated the development of tissue engineering strategies to repair cartilage lesions before they progress to osteoarthritis. Despite an increasing body of research focusing on the treatment of cartilage defects, common strategies including autologous chondrocyte implantation (ACI) and matrix-induced autologous chondrocyte implantation (MACI), often fail to promote hyaline cartilage repair. This may be due, at least in part, to the fact that the chondrocytes used during these treatments undergo de-differentiation during monolayer expansion, compromising the quality of the resulting tissue. Bone marrow derived mesenchymal stem cells (MSC) are a promising alternative cell source for cartilage tissue engineering due to their ease of isolation and capacity to undergo rapid in vitro expansion. The ability to regulate their differentiation is crucial to the success of MSC based tissue engineering therapies. While it is well known that MSC differentiation can be regulated by both soluble (i.e. growth factors) and insoluble factors (i.e. substrate rigidity and cell morphology), how such factors interact to regulate chondrogenesis of adult stem cells remains relatively poorly understood.

Both cell morphology and matrix stiffness have been shown to determine MSC fate. The mechanism by which cell morphology and actin
cytoskeleton influence MSC fate has been explored using a system of topographies that promote either a spread cell shape, which is generally correlated with osteogenesis, or topographies that promote a round MSC shape, generally supporting adipogenesis. This shape change from a rounded morphology to a flattened morphology influences the architecture of the actin cytoskeleton, suggesting that cells respond to topographical cues at least partially by modulating Rho/Rock pathways. A spread MSC morphology is generally correlated with an inhibition of chondrogenesis, which can be prevented by restricting cell attachment and cytoskeletal tension.

Cells that adhere to a substrate have been shown to exert contractile forces, resulting in a tensile stresses in the cytoskeleton. The interplay of these forces and the stiffness of the substrate can have a robust impact on cell behavior such as migration, apoptosis and proliferation. In hydrogel systems (non-degradable, ionically crosslinked alginate hydrogels), encapsulated MSC differentiation is dictated by matrix stiffness irrespective of cell morphology. It is generally accepted that stiffer environments influence MSC toward an osteogenic phenotype, whilst softer ones are more adipogenic. Matrix stiffness has also been shown to regulate chondrogenesis, with some studies demonstrating that softer substrates are more supportive of chondrogenesis, while other show that substrates mimicking the stiffness of articular cartilage are more chondrogenic. Although recent studies have demonstrated that hydrogel stiffness regulates cartilage matrix formation by chondrocytes, the impact of hydrogel elasticity on MSC chondrogenic differentiation remains poorly understood.

To experience the mechanical inputs arising from their microenvironment, cells generate cytoskeletal contractile forces to actively feel their surroundings. The major actuator of the Hippo pathway, the YAP/TAZ complex, is thought to be a
cellular rheostat able to interpret mechanical signals, such that cytoskeletal tension or cell growth on stiff hydrogels promotes YAP nuclear entry and activity, while cell-cell interactions or soft hydrogels cause its cytosolic retention\textsuperscript{24,124,125,182–184}. Lee \textit{et al.}\textsuperscript{185} have recently shown that in breast cancer patient samples and in a model of 3D mechanically tunable \textit{in vitro} cell cultures, mechanotransduction happens independently of YAP. Similarly, a study on the osteogenic differentiation of MSC showed that cell volume expansion triggered RUNX2 nuclear entry and activity, but not YAP\textsuperscript{176}. So while the role of YAP in 2D culture environments is well established\textsuperscript{82,83,148}, its function in more physiological relevant 3D environment remains more poorly understood. Furthermore, the regulatory role that YAP might plays in mechanotransduction during chondrogenesis of MSC requires further investigation.

Dimensionality (i.e. 2D versus 3D culture) is another key determinant of stem cell chondrogenesis. 2D cell culture environments (e.g. tissue culture plastic) are generally not representative of the \textit{in vivo} environment, with 3D environments (e.g. hydrogel encapsulation) known to better support different physiological cellular functions, from differentiation to maintenance of stemness\textsuperscript{14}. Differences in chondrogenic gene expression of articular chondrocytes in 2D versus 3D cultures have been reported\textsuperscript{186}. The effects of dimensionality have been observed also across other cell types\textsuperscript{187–189}. A stark difference determined by dimensionality can be seen in the apical-basal polarity of cells grown in 2D, which is fixed by a solid support below and the medium above, while cells growing in 3D experience the ECM in every direction. The binding of the ECM on the apical domain has been shown to trigger different responses than the basal domain, in terms of gene expression or cytoskeletal organization\textsuperscript{190–192}. The additional dimensionality of 3D cell culture cannot be described as a single variable, thus in order to examine its role, cells
grown in 2D need to be directly compared to cells embedded within the same material.

Natural polymers such as agarose, alginate and type I collagen are often used for 3D cell culture or as cell-laden biomaterial implants to promote tissue regeneration\textsuperscript{70,86,193,194}. Such materials don’t always allow to decouple the effect of stiffness from scaffold architecture or ligand density. These limitations can be overcome by the use of hydrogels made of interpenetrating networks (IPN), which consist of two or more interlaced components\textsuperscript{16,17,118,119}. In this study type I collagen, which provides intrinsic cell-adhesive sites, and alginate, a polysaccharide with tunable mechanical properties, were combined to form an IPN hydrogel system\textsuperscript{17,89,195}. MSC can either be grown on the surface of these hydrogels (2D culture; Chapter 4) or encapsulated within the hydrogel (3D culture). Furthermore, the stiffness of the IPN hydrogel, as well as the morphology of the cells embedded in the hydrogel, can be independently tuned by altering the extent and timing of hydrogel cross-linking.

The aim of this study was to explore how substrate stiffness and cell morphology regulate chondrogenesis of MSC within these collagen-alginate IPN. The first hypothesis is that MSC encapsulated within IPN hydrogels (3D culture) would undergo more robust chondrogenesis than MSC cultured on the surface of the same hydrogels (2D culture). The second hypothesis of this study is that encapsulation of MSC into soft hydrogels that support a more rounded cell morphology would better support chondrogenesis. To test these hypothesis, MSC were cultured both within the 3D IPN and on top of the soft 2D IPN in presence of the chondrogenic factor TGF-β3. Next the interplay between substrate stiffness and cell morphology on the chondrogenic commitment of MSC encapsulated within these hydrogels was evaluated by determining the expression of key chondrogenic
genes and the cellular localization of different regulatory proteins. Contrary to our initial hypothesis, hydrogels that initially allowed MSC to spread within a hydrogel better supported chondrogenesis, which could be linked to differences in cell volume expansion and cell-cell interactions observed in the different hydrogels.
5.2 Materials and Methods

5.2.1 3D IPN fabrication and culture.

The collagen-alginate solution was prepared as described in the previous chapter. Briefly 1 mL of 6% ice-cold collagen type I solution (from rat tail, Corning) had the latter addition of 400 µl of 10X RPMI (Merck), 350 µl of collagen neutralization buffer containing 0.1M HEPES and 1M sodium bicarbonate dissolved in PBS to reach pH 7.4. At this point, a volume of 400 µl of MSC previously trypsinized and resuspended in GM (5 million MSC/ml), was gently mixed to the neutralized collagen solution. Finally, 2 ml of 3.5% alginate solution (UP LVG, batch# BP-0907-02, viscosity = 198 mPa*s, Pronova matrix) were thoroughly mixed, pipetted into a custom-made mould (Figure 5-1a) and placed in incubation. The mould was composed of the following main parts: two metal plates, two dialysis membranes and a silicon slab. Both metal plates and the silicon slab had perforations that needed to be aligned to allow homogenous diffusion of crosslinker through the constructs. As shown in Figure 5-1a, the cell-biomaterial solution was initially pipetted into a half-assembled mould, which was then completed by a second layer of dialysis membrane and the last metal plate. The mould was then placed in a sterile case made for this purpose (as shown in the schematic of Figure 5-1a) or in a petri dish containing silicon pedestals (cylinders of 8 mm of diameter by 2 mm of thickness) to hold in suspension the assembled mould and allow the crosslinker to fully cover all the surfaces. At this point, to tailor the morphology of the MSC, the mould was incubated for either 10 minutes (round cell shape, also termed “immediate crosslinking”) or 4 hours (spread cell shape, also termed “delayed crosslinking”) before the addition of the crosslinker (hgDMED (Gibco) containing 20 mM CaCl₂ (Merck)). Finally, the elasticity of the IPN was tuned by exposing
the samples to the crosslinker for either 40 minutes (soft 3D IPN) or 150 minutes (stiff 3D IPN). After the incubation time, the 3D IPN samples were removed from the mould, rinsed in fresh hgDMEM and incubated in GM for 12 hours. Then, GM was replaced with starvation media composed of hgDMEM, penicillin (100 U/mL) and streptomycin (100 mg/mL) and 0.5% FBS (Gibco) for 24 hours, to allow cell cycle synchronization, later replaced by CDM. Samples were cultured for 7 days at 5% oxygen, in a humidified atmosphere at 37 °C and 5% CO₂.
5.3 Results

5.3.1 Maintaining MSC in 3D environments better supports chondrogenesis

An IPN hydrogel consisting of collagen type I and alginate was developed to examine the effect of dimensionality (2D versus 3D culture) and the interplay between substrate stiffness and cell morphology on chondrogenesis of MSC. Matrix elasticity could be tuned by adjusting the duration of exposure to the alginate crosslinker (CaCl$_2$), while cell morphology could be modulated by delaying the crosslinking of the alginate hydrogel (Figure 5-1a). It was possible to increase the 3D IPN elasticity from 5.2±0.7 kPa (soft) to 17.5±1.8 kPa (stiff) (Figure 5-1b) without impairing cell viability (Figure 5-1c). The range of stiffness achieved was comparable to the 2D IPN described in the previous chapter (see also Appendix II, Figure S-0-2 for direct comparison of 2D and 3D IPN mechanical properties). Following encapsulation of MSC into the hydrogel (3D culture), the immediate crosslinking of the alginate network forced the cells to adopt a rounded morphology (Figure 5-1d), while delaying alginate crosslinking (4 hours of incubation) allowed the cells to assume a spread shape by allowing them to sense and adhere to the collagen fibres (Figure 5-1d). MSC that were allowed to spread in a soft 3D IPN possessed a higher volume when compared to cells constrained to a round morphology in a hydrogel of the same stiffness (Figure 5-1e), while the volume of round and spread MSC didn’t change when they were kept in the stiff 3D IPN (Figure 5-1e).
Figure 5-1 | 3D IPN characterization and validation. a) Schematic depicting the 3D IPN fabrication procedure. b) 3D IPN Mechanical properties, ****p<0.0001. e) Confocal analysis of MSC viability showing live cells (green) and dead cells (red). Scale bar, 100 µm. d) Confocal analysis of MSC growing into 3D IPN. In particular, magnification of single cells. Cells were stained for actin (magenta) and nuclei (blue). Scale bars 100 µm and 10 µm. Graph:
sphericity of single cells, ****p<0.0001. e) Graph reporting the volume of MSC embedded in the 3D IPN, **p<0.01.

To examine the effect of dimensionality (2D versus 3D culture) on chondrogenesis of MSC, the expression of SOX9, ACAN and COL2 was compared for MSC cultured on the surface of soft IPN (2D culture) or encapsulated within the same soft IPN (3D culture). MSC encapsulated within the IPN were allowed to adopt a spread morphology, as MSC cultured on the surface of the IPN also adopt a spread morphology. The expression of SOX9, ACAN and COL2 was significantly higher in MSC maintained in a 3D environment (Figure 5-2a). MSC maintained in the more physiologically relevant 3D environment were more spherical and possessed a smaller volume (Figure 5-2b).
Figure 5-2 | Dimensionality influences MSC chondrogenesis. a) Gene expression analysis of MSC cultured for 7 days on top of soft 2D IPN or into soft 3D IPN with a spread morphology, reported as relative to 2D soft group ***p<0.001 b) Confocal imaging of MSC cultured for 7 days on top of soft 2D IPN (left) or into soft 3D IPN with a spread morphology (right). Cell stained for actin (magenta) and nuclei (blue). Scale bar 10 µm. Graphs: quantification of sphericity and volume of MSC growing in a 2D or 3D (spread morphology) soft IPN, ****p<0.0001.

5.3.2 Soft hydrogels that facilitate cellular spreading better support chondrogenesis of MSC

Having determined the impact of dimensionality, the interplay of matrix stiffness and cell morphology on chondrogenesis of MSC following encapsulation
within the hydrogels (i.e. 3D culture) was next examined. MSC were encapsulated within the 3D IPN and cultured for 7 days in presence of chondrogenic growth factors. Irrespective of the matrix stiffness, MSC that were initially allowed to adopt a spread morphology expressed higher levels of SOX9, ACAN and COMP(Figure 5-3a). Matrix stiffness also regulated chondrogenesis of MSC, with those maintained in soft environments and allowed to spread expressing higher levels of COL2 and COLX compared to spread MSC in stiff hydrogels (Figure 5-3a). In agreement with the mRNA expression profile, type II collagen protein synthesis was higher in the soft IPN that allowed spreading of MSC (Figure 5-3b). COL2 expression showed to correlate with the overall morphology of the cells (Figure 5-3c), with spread cells expressing higher amount of the protein, however this interdependency correlated to a greater extent when the COL2 fluorescent intensity was analysed against cell volume increase (Figure 5-3d).
Figure 5-3 | Gene expression of chondrogenic markers. 

a) Analysis of gene regulation in MSC embedded in 3D IPN and cultured for 7 days in chondrogenic media. mRNA levels reported as fold change normalised to rounded cells into soft 3D IPN. *p<0.05, **p<0.01, ****p<0.0001.

b) Confocal analysis for COL2 production, cell stained for COL2 (red) and nuclei (blue). Scale bar 100 μm.
Graph reporting the average percentage of cells positive for COL2 staining. **p<0.01. c) Correlation analysis of COL2 intensity and cell shape index or d) volume after 7 days of culture.

SMADS are the main signal transducers for receptors of the transforming growth factor beta (TGF-β) superfamily, with SMAD 2/3 signalling key to normal cartilage development. Subtle differences in the levels of nuclear SMAD 2/3 were observed in the different hydrogel systems, with enhanced nuclear localization of SMAD 2/3 observed in the softer hydrogels that facilitated initial cell spreading (Figure 5-4).
Figure 5-4 | Spreading of MSC in a soft 3D IPN drives SMAD2/3 nuclear localization. Confocal analysis of MSC embedded into 3D IPN and cultured for 7 days in chondrogenic conditions. Cells stained for actin (magenta), SMAD2/3 (green) and nuclei (blue). Scale bar, 10µm. Graph: nuclear quantification of SMAD 2/3. *p<0.05, **p<0.01, ****p<0.0001

The expression of NCAD, a transmembrane protein that functions to mediate cell–cell adhesions, appeared unaffected by the initial shape of the MSC in the soft IPN. In the stiff IPN, higher levels of NCAD were observed around the spread MSC compared to the round MSC (Figure 5-5).
Figure 5-5 | Round MSC growing in a soft 3D IPN secrete more NCAD.

Representative confocal images of MSC stained for actin (magenta), NCAD (grey) and nuclei (blue). Scale bar, 10 µm. Graph: quantification of total NCAD staining. ***p<0.001, *p<0.05
5.3.3 *Enhanced chondrogenesis is correlated with higher cell volumes and increases in cellular aggregation.*

To assess how MSC respond to the 3D IPN in determining their chondrogenic fate, morphology (*sphericity*), cell volume and cell-cell interaction (as determined by measuring cell-cell distances) were examined over time. Beside the need to tailor matrix stiffness, the 3D IPN was designed to initially instil a defined MSC morphology, while leaving the cells free to rearrange the matrix over the culture duration. Initially MSC encapsulated in the softer hydrogels and allowed to spread saw their volume increase by almost 3-fold compared to MSC forced to initially maintain a rounded morphology, while MSC encapsulated in the stiffer hydrogels had comparable initial volumes irrespective of their initial shape (Figure 5-6a). Although significant volume changes occurred over time, spread MSC maintained in soft hydrogels remained larger over the duration of the culture period (Figure 5-6a).

To quantify the extent of cell-cell interactions within the different hydrogels, the distance between MSC was measured over time in culture. At day 1, shorter cell-cell distances were observed in the softer hydrogels when MSC were allowed to initially spread, with comparable cell-cell distances observed in the stiffer hydrogels irrespective of their initial morphology (Figure 5-6b). At day 3 in the stiffer gels, MSC that were initially spread were found to be closer to each other than MSC initially forced to adopt a rounded morphology. At the end of the 7 day culture period, the initially rounded MSC cultured in the stiff 3D IPN were found to have the shortest cell-cell separation (gap of $45.6 \pm 25.9 \, \mu m$), with the largest cell-cell distance measured in similarly shaped cells maintained in a softer matrix (gap of $55.1 \pm 28.3 \, \mu m$) (Figure 5-6b). At day 7, the initially spread MSC that were maintained in a soft matrix were in closer proximity ($49 \pm 28.2 \, \mu m$) than those kept
in the stiffer 3D IPN (52.6 ± 29 µm) (Figure 5-6b). To be noted, the calculation of the average cell-cell distance excluded cells forming aggregates, therefore since contacting cells have a distance =0, it is possible to assume that the average cell distance might have been overestimated.

The extend of cellular aggregation was also assessed. MSC that were initially allowed to spread aggregated to a greater extent that MSC that were initially rounded, with the highest levels of cellular aggregation observed in the soft hydrogels. Examination of cell distribution showed that among all the objects (single cells and interacting cells) detected within the soft-spread group, more than 30% of those were found to be interacting cells forming aggregates (Figure 5-6c).
Figure 5-6 | A soft 3D IPN that allows MSC spreading drives cell aggregation. a) Graphs showing cell sphericity and volume at day 1, 3 and 7. *p<0.05, **p<0.01, ****p<0.0001. b) Graph reporting cells average distance over time. The scale of the images is 3.5214 px/µm and it can be used to convert the px values into µm. *p<0.05, ****p<0.0001. c) Confocal analysis of MSC
cultured in 3D IPN for 7 days. Cells stained for actin (magenta) and nuclei (blue). Scale bar, 100 µm. Graph reporting the average percentage of aggregates per field of view. **p<0.01, ****p<0.0001.

5.3.4 Hindering cell spreading inhibits the chondrogenic differentiation of MSC.

To understand whether the interplay of matrix stiffness and cell morphology was linked with an altered cellular signalling, YAP localization (whose role in chondrogenesis is controversial\textsuperscript{82,83,145} and osteogenic gene expression were next examined. YAP nuclear localization was found to be more abundant in initially rounded MSC maintained for 7 days in a stiff 3D IPN (Figure 5-7a). The expression of YAP mRNA levels was higher in initially rounded MSC irrespective of matrix stiffness (Figure 5-7b). The expression levels of RUNX2 and COL1 genes were highest in initially rounded MSC maintained in soft hydrogels (Figure 5-7b). The ratio of SOX9/RUNX2 expression, which is commonly used as a marker of stable chondrogenesis\textsuperscript{196}, further confirmed that facilitating the initial spreading of MSC enhanced chondrogenic differentiation (Figure 5-7b).
Figure 5-7 | A soft 3D IPN inhibits rounded MSC to differentiate toward chondrogenesis. a) Confocal analysis of MSC cultured for 7 days into 3D IPN. Cells stained for YAP (yellow), actin (magenta) and nuclei (blue). Scale bar, 10 μm. Graph: YAP nuclear quantification. ****p<0.0001, b) Gene expression analysis of MSC at day 7 relative to soft round group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
5.4 Discussion

The primary objective of this study was to examine the interplay between matrix elasticity and cell morphology on chondrogenesis of MSC following encapsulation within collagen-alginate hydrogels. Encapsulation of MSC within a hydrogel (3D culture) greatly enhanced chondrogenesis compared to culture of the same cells on the surface of the hydrogel (2D culture). Contrary to what was initially hypothesised, allowing the cells to spread within a soft hydrogel led to more robust chondrogenesis. In such conditions, cells could form more aggregates and expressed higher levels of key chondrogenic markers at the gene and protein level. This was associated with enhanced SMAD 2/3 nuclear entry and lower levels of nuclear YAP compared to other hydrogel environments. In contrast, forcing MSC to initially maintain a rounded morphology and a lower cell volume in the softer hydrogels led to increased expression of osteogenic markers (Figure 5-8).
Figura 5-8 | Cells grown into a soft 3D IPN express key markers of chondrogenesis.

MSC encapsulated within the hydrogel underwent more robust chondrogenic differentiation than cells kept on the hydrogel surface. As discussed in the previous chapter, the IPN system was chosen because its versatility in allowing MSC growth in 2D and 3D. In this chapter the fabrication procedure was adapted to tailor not only matrix elasticity, but also cell morphology in 3D. It is known that cell morphology and signalling are significantly altered by culture dimensionality\textsuperscript{64,197}, with a 3D environment critical to chondrogenesis\textsuperscript{198}. Indeed, a common way to evaluate the chondrogenic potential of MSC is the pellet model, which provide a 3D environment that allows cell–cell interactions similar to those observed in pre-cartilage condensations found during embryonic development\textsuperscript{2}. It has been found that the switch from 2D to 3D culture, within the same media supplementation, facilitated a change from osteogenesis to terminal chondrogenesis\textsuperscript{198}. In this case, osteogenesis was more pronounced when MSC were cultured on top of stiff gels (~30 kPa), while their encapsulation within the
same stiff matrix caused the cells to undergo terminal chondrogenesis\textsuperscript{198}, demonstrating the fundamental role and impact of culture dimensionality on lineage commitment.

Chondrogenesis was favoured by a soft hydrogel that facilitated the initial spreading of MSC. Such conditions lead to a higher cell volume and enhanced the nuclear translocation of SMAD 2/3, the upregulation of chondrogenic genes (SOX9, \textit{ACAN}, \textit{COL2}, \textit{COMP COLX}) and increased type II collagen protein synthesis. The SMAD 2/3 complex is a direct signal transducer of the TGF-\(\beta\) signalling pathway, which is one of the most prominent growth factors involved in the chondrogenic differentiation of MSC\textsuperscript{135,136,138}. Upon activation, SMAD 2/3 translocate into the nucleus where they can regulate the transcription of target genes, i.e. SOX9. By direct binding of \textit{COL2} DNA sequence, SOX9 controls \textit{COL2} mRNA levels, which together with aggrecan (\textit{ACAN}) and cartilage oligomeric protein (\textit{COMP}) are fundamental constituents of the cartilage ECM\textsuperscript{155,156}. Although type X collagen (\textit{COLX}) is commonly used as a marker of late stage chondrocyte hypertrophy, its expression is also observed during the early stages of chondrogenesis and it should not be exclusively viewed as a marker of progression along the endochondral pathway\textsuperscript{199–202}.

Hydrogel conditions that supported higher levels of chondrogenesis were not associated with higher levels of NCAD expression. In general, the cadherins family are a group of \(\text{Ca}^{2+}\) dependent, single transmembrane glycoproteins that mediate cell–cell adhesion by homotypic protein–protein interactions through their extracellular domain, and through their cytoplasmic domain, bind the actin cytoskeleton by means of the catenin family of proteins\textsuperscript{38}. NCAD is expressed in the developing embryonic limb bud in a spatiotemporally specific manner mediating cellular condensation\textsuperscript{103,120,199}. In the central core of the cartilage anlage,
NCAD expression increases as condensation begins and diminishes when the chondrogenic differentiation starts, thus implying that MSC must undergo a decrease in cell–cell adhesion following cell condensation. NCAD presence during the chondrogenic development is not only regulated spatiotemporally, but also its dosage was found to be another important parameter driving cell chondrogenesis. Stably transfected C3H10T1/2 cells expressing NCAD shown that a 2-fold increase of the protein enhanced chondrogenesis, while a 4-fold increase in NCAD resulted in an inhibitory effect on chondrogenesis. These findings demonstrate that the role of NCAD in chondrogenesis is complex and must be interpreted with caution.

The higher levels of chondrogenesis observed with the spread MSC maintained in soft hydrogels was correlated with the formation of cell aggregates/colonies. As no significant difference in cell proliferation between the experimental groups and compared to day 0 (see Appendix IV, Figure S-0-3), this cellular aggregation was likely due to increased motility of the cells thorough the soft 3D matrix. In the context of tissue development, chondrogenesis is initiated by cell-cell adhesion. Such interactions are mediated by N-cadherin (NCAD), a transmembrane protein considered fundamental for chondrogenesis to occur. In developing limb buds, NCAD mediated cell condensation is followed by SOX9 transcription and cartilage matrix anabolism. Successful cartilage tissue engineering will likely require the development of hydrogel environments that recapitulate key aspects of cartilage development.

The same hydrogel environment that elicited higher levels of chondrogenesis also supported an increase in cell volume. It has been demonstrated that the volume expansion of cells cultured into fast stress-relaxing hydrogels (~20 kPa) is associated with osteogenic differentiation. While the growth of chondrocytes in hydrogels (~3 kPa) in which the stresses are slowly relaxed over
time, restricted cell volume expansion and therefore enhanced the secretion of interleukin-1β, which in turn drove the expression of genes associated with cartilage degradation and cell death\textsuperscript{73}. In the case of this thesis, a volume oscillation is seen, in which cells initially bigger in size, restricted at day 3, and then increase again their volume at day 7. Developmentally, the chondrocytes in the central region of the cartilage anlage can increase their volume up to 20-fold when shifting to an hypertrophic phenotype\textsuperscript{26,102,204}. Although not directly proven, it is possible that the enhanced chondrogenic response of MSC in the soft 3D IPN might have been similarly regulated by the synergy of cell morphology and volume expansion oscillation over the culture period.

This study also demonstrated that osteogenic differentiation of MSC is enhanced by a soft 3D IPN restricting cell morphology to a rounded shape. Contrary to previous 2D studies reporting the association between increased YAP activation and osteogenesis\textsuperscript{24,147,205}, YAP nuclear translocation didn’t correlate with the upregulation of \textit{RUNX2} and \textit{COL1} in this study, although an increased level of \textit{YAP} mRNA was found in round MSC kept at both stiffness. YAP activity in a cell is under direct control of its shape, which is in turn dictated by the cytoskeletal structure (i.e. F-actin network and contractility)\textsuperscript{125,206}. Here, the F-actin network of the spread cells, although examined only qualitatively, seemed to be mostly distributed cortically to the cells. Indeed, it is known in literature that cells cultured in 3D fails to form robust stress fibres, preferring a cortical distribution of F-actin\textsuperscript{185,207,208}, possibly explaining why YAP behaviour in 2D and 3D differs\textsuperscript{176,185,209}.

Although this study described a possible mechanism through which MSC respond to the interplay of substrate elasticity and cell morphology under chondrogenic conditions, we explored only partially the role of dimensionality. Our
data showed that MSC were able to feel and interact with the 3D IPN and although being outside the aim of the study, an investigation of the specific forces exerted on the matrix could give more insights on the possible mechanisms of mechanotransduction triggered by such 3D environment.

In conclusion, this chapter described the chondrogenic differentiation of MSC in response to TGF-β3 stimulation as function of the substrate mechanical properties and initial cell morphology. In particular, this study demonstrated that a physiologically relevant environment that approaches in vitro the stiffness and cell morphology of the developing cartilage tissue, enhances the chondrogenic potential of MSC, partially by supporting volume expansion and cellular aggregation. The results described in this work evidenced the fundamental role of biophysical cues in regulating MSC biology and the importance of this information to inspire new strategies for stem cell based cartilage regeneration procedures.
Chapter 6  The interplay between hydrostatic pressure and matrix stiffness in regulating chondrogenesis of mesenchymal stem cells

6.1 Introduction

The avascular, aneural and alymphatic nature of cartilage tissue hinders its ability to self-repair, leading to progressive joint damage\textsuperscript{210}. To date, neither conventional cartilage repair treatments such as microfracture or autografts, or regeneration strategies such as autologous chondrocytes (ACI)\textsuperscript{211} can predictably restore the damaged tissue to its original state. Major limitations of these applications include donor-site morbidity, lack of integration and dedifferentiation of chondrocytes during \textit{in vitro} expansion\textsuperscript{212–214}. Tissue engineering strategies that aim to recapitulate aspects of mesenchymal condensation and cartilage development represent promising new approaches for joint regeneration\textsuperscript{210,215,216}. Developmentally, cartilage formation begins with mesenchymal condensation leading to chondrogenic differentiation of mesenchymal cells\textsuperscript{217}. In response to cell condensation, a dense matrix is produced, serving as a cartilage anlage, which will lead to the formation of both articular cartilage and subchondral bone\textsuperscript{218}. In the context of a developmentally inspired strategy for cartilage tissue engineering, mesenchymal stem/stromal cells (MSC) represent a promising cell source due to their ease of isolation and expansion and capacity to give rise to different musculoskeletal tissues\textsuperscript{216,219}. MSC differentiation depends on cues present on the local environment, and while much attention has focused on soluble factors to direct their chondrogenic differentiation, less attention has been given to physical stimuli such as substrate rigidity and external mechanical forces\textsuperscript{57,161,164,220,221}. 

\textsuperscript{110}
Lineage commitment of MSC can be regulated by the elasticity of the substrate and its topography.\textsuperscript{5,8,170,222–225} Engler \textit{et al.}\textsuperscript{5} reported that 2D substrates mimicking the stiffness of brain tissue elicited a neurogenic response, stiffer substrates akin to skeletal muscle induced cells to undergo myogenesis, while stiffer substrates matching the rigidity of the developing bone drove the cells towards osteogenesis. These studies have typically explored the role of substrate stiffness on MSC fate in 2D culture systems,\textsuperscript{112,115,226} however the role of matrix stiffness in directing differentiation in a 3D environment is more complex. Using an ionically crosslinked and RGD-modified alginate hydrogel model, it has been demonstrated that stiffer gels promoted osteogenesis and softer adipogenesis.\textsuperscript{70} In this 3D hydrogel system, matrix stiffness did not impact cell morphology, with MSC maintaining a rounded shape irrespective of their local stiffness. Hydrogel stiffness did however determine integrin binding as well as reorganization of adhesion ligands, which correlated with osteogenesis of MSC.\textsuperscript{70} Hydrogel degradation has also been shown to regulate the differentiation of MSC. In non-degradable hydrogels, MSC underwent adipogenesis over a large span of stiffness (4.91 – 91.64 kPa), but when cells were allowed to degrade the matrix (hydrogel stiffness = 3-5 kPa), then they could generate cytoskeletal tension and differentiate down an osteogenic lineage.\textsuperscript{25} In the context of chondrogenesis, a stiffness mimicking the rigidity of healthy articular cartilage (0.5 MPa) has been shown to enhance the expression of \textit{SOX9}, \textit{ACAN} and \textit{COL2} in primary chondrocytes and ATDC5 cells (chondrogenic cell line) grown onto 2D substrates. This study also found that substrate stiffness also determines the response of such cells to exogenous growth factors, with the appropriate combination of ECM stiffness and TGFβ stimulation inducing chondrocyte gene expression more robustly than either cue alone.\textsuperscript{68} Other studies have suggested that much softer substrates (~1kPa) are more supportive of
human stem cell chondrogenesis. Substrate stiffness has also been found to regulate chondrogenesis of human MSC in an oxygen concentration dependent manner. Physioxia upregulated chondrogenesis to a greater extent when cells were cultured on relatively soft substrates (0.167 kPa), a stiffness approaching that of the developing cartilage anlage. This uncertainty in the literature warrants further investigation into the role played by matrix stiffness in regulating chondrogenesis of MSC in physiologically relevant 3D environments.

Biophysical stimuli such as compression and hydrostatic pressure (HP) have also been shown to regulate chondrogenesis of stem cells. In vivo, the removal of physical cues has been associated with the arrest of embryogenesis. Thus, in order to drive development, mechanical cues must be presented in an appropriate spatial and temporal manner in combination with biochemical gradients. The response of MSC to both biochemical and biophysical cues is complex, for instance the simultaneous application of TGF-β and dynamic compression during the early chondrogenic differentiation resulted in a suppression of chondrogenic genes and matrix accumulation. Hydrostatic pressure, a loading modality that results in little or no cellular deformation, is also key regulator of chondrogenesis. HP has been shown to play a role in regulating chondrogenic differentiation during limb development, while the application of physiological frequencies and magnitudes of HP (up to 12 MPa) has been shown to increase cartilage matrix synthesis and regulate hypertrophy. In the presence or absence of exogenous TGF-β, cyclic HP has been shown to enhance chondrogenesis of MSC at gene and/or protein level. The response of MSC to HP has also been shown to depend on the stiffness of their surrounding substrate. Understanding the interplay between matrix stiffness and extrinsic mechanical cues such as HP will
be critical to the development of tissue engineering strategies aiming to use MSC to generate stable articular cartilage.

Despite HP emerging as a key mechanical stimuli for development and maintenance of articular cartilage, tissue engineering strategies that successfully integrate this regulatory cue have yet to be established\(^{233}\). The goal of this study was to investigate how HP, substrate stiffness and TGF-β3 interact to regulate chondrogenesis of MSC. To this end, this study examined the combined effect of HP and substrate stiffness in presence or absence of TGF-β3 on the chondrogenic commitment of MSC seeded collagen/alginate interpenetrating networks (3D IPN) hydrogels. The initial hypothesis of this study was that HP, with or without TGF-β3 supplementation, would enhance chondrogenesis of MSC embedded in a soft 3D IPN, while it would rescue the chondrogenic phenotype in MSC seeded in the stiffer matrix. This study found that HP stimuli in absence of exogenous TGF-β3 does not promote chondrogenesis of MSC. The early application of HP, in presence of TGF-β3, inhibited the condensation of MSC and supressed the expression of key chondrogenic markers. In contrast, the delayed application of HP, in presence of TGF-β3, promoted MSC condensation and enhanced the expression of chondrogenic genes in MSC grown in the stiffer hydrogels. These findings demonstrated how external mechanical cues can be leveraged to regulate chondrogenesis of MSC and opens alternative avenues for developmentally inspired strategies for cartilage tissue regeneration.
6.2 Materials and Methods

6.2.1 3D IPN fabrication and culture.

The collagen-alginate solution was prepared as described in the previous chapter. Briefly 1 mL of 6% ice-cold collagen type I solution (from rat tail, Corning) had the latter addition of 400 µl of 10X RPMI (Merck), 350 µl of collagen neutralization buffer containing 0.1M HEPES and 1M sodium bicarbonate dissolved in PBS to reach pH 7.4. At this point, a volume of 400 µl of MSC previously trypsinized and resuspended in GM (5 million MSC/ml), was gently mixed to the neutralized collagen solution. Finally, 2 ml of 3.5% alginate solution (UP LVG, batch# BP-0907-02, viscosity = 198 mPa*s, Pronova matrix) were thoroughly mixed, pipetted into a custom-made mould and placed in incubation for 4 hours to allow cell spreading before the addition of the crosslinker (hgDMED (Gibco) containing 20 mM CaCl₂ (Merck)). Finally, the elasticity of the IPN was tuned by exposing the samples to the crosslinker for either 40 minutes (soft 3D IPN) or 150 minutes (stiff 3D IPN). After the incubation time, the 3D IPN samples were removed from the mould, rinsed in fresh hgDMEM and incubated in GM for 12 hours at 5% oxygen, in a humidified atmosphere at 37 °C and 5% CO₂.

6.2.2 Application of hydrostatic pressure

The schematic in Figure 6-1a shows the preparation of the samples for the HP loading experiments. Cell seeded 3D IPN (see Chapter 5 for details on 3D IPN fabrication) were prepared and left to equilibrate overnight in GM. The next day, the 3D IPN samples were encapsulated, evenly spaced, in a 2% agarose (A2790, Merck) block to provide protection from handling damage and inserted into heat-sealed, gas-permeable, water-tight, sterile bags (EVO120, Quest Biomedical, UK)
with 3 mL of medium per construct, removing the air via a needle free port. Before the loading experiment, samples were incubated for 24 hours in starvation media (hgDMEM, penicillin (100 U/mL) and streptomycin (100 mg/mL) and 0.5% FBS (Gibco)) for cell cycle synchronization. Cyclic HP was applied via a water filled, custom-made bioreactor within a 37°C incubator as described previously. The sealed bags exposed to HP were placed into the pressure vessel, while the free swelling controls were placed into an open water bath next to the pressure vessel. HP was applied at an amplitude of 2 MPa and a frequency of 1 Hz for a duration of 4 hours per day, for 7 consecutive days (or 14 days when specified). The bags were returned to a culture incubator (37°C, 5% CO₂, 5% O₂) between loading periods and suspended separately in an upright position for homogenous gas transfer.

6.2.3 Experimental design

Figure 6-1b describes the sequential order of the experiments herein reported. Firstly, a loading experiment in the absence of chondrogenic factors was carried in order to evaluate the chondro-inductive nature of HP stimulation. Next, MSC seeded constructs were cultured in chondrogenic medium containing TGF-β3 for 7 days in presence of cyclic HP as described above. In the last study, samples were cultured in free swelling conditions in chondrogenic medium (containing TGF-β) for 1 week, followed by 1 week of culture in the same TGF-β3 containing medium where HP stimulation was applied as described above. All study groups consisted of a hydrostatically loaded group (HP) and a free-swelling unloaded control (FS).
6.3 Results

6.3.1 Hydrostatic pressure alone does not initiate chondrogenesis of MSC

To better understand the role of HP in regulating the initiation and progression of chondrogenesis, MSC were seeded into soft (5.2 ± 0.7 kPa) or stiff (17.5 ± 1.8 kPa) 3D IPN hydrogels and subjected to HP stimulation. To facilitate construct handling during the loading phase, the 3D IPN samples were embedded into agarose blocks, placed into cell-culture bags and loaded at a frequency of 1Hz for 4 h/day at 2MPa (Figure 6-1a). A series of studies were conducted in absence or presence of TGF-β for 7 days (Figure 6-1b, 1 and 2), while in the final study MSC were cultured in free swelling chondrogenic conditions for one week, followed by another week of HP stimulation in presence of the same chondrogenic factors (Figure 6-1b, 3). The HP handling and loading didn’t cause any detrimental effect on cell viability (Figure 6-1c, day 7 +TGF-β and day 14 not shown). HP stimulation in absence of TGF-β was not observed to promote chondrogenesis of MSC (Figure 6-1d). Only COL1 expression was found to be upregulated in stiff gels following the application of HP. As no increases in the cartilage specific markers SOX9, ACAN and COL2 were observed with the application of HP in absence of exogenous TGF-β, the interaction between the application of HP and exogenous TGF-β was next examined (Figure 6-2).
Figure 6-1 | HP loading is not enough to induce the chondrogenic differentiation of MSC. Schematics depicting a) the samples preparation procedure and b) the experimental design. c) Qualitative analysis of MSC viability after 7 days of dynamic culture in absence of TGF-β. Live cell stained in green, dead cells in red. Scale bar 1 mm. d) Gene expression analysis of MSC
embedded into 3D IPN and dynamically cultured for 7 days in absence of chondrogenic factors. NE: not expressed. *p<0.05, **p<0.01, ****p<0.0001.

As observed previously, the softer hydrogels better supported a chondrogenic phenotype in free swelling conditions, as evident by higher levels of ACAN and COL2 expression (Figure 6-2). Hydrostatic pressure was found to reduce the expression of ACAN, COL2, CDH2 and COLX in MSC encapsulated within the softer IPN hydrogels (Figure 6-2). In the stiffer hydrogels, hydrostatic pressure reduced the expression of CDH2, COLX and TGFBR2 (Figure 6-2). The already low levels of ACAN and COL2 expression in the stiff hydrogels were not further reduced by the application of HP.

**Figure 6-2 | Inhibitory effect of HP on chondrogenesis of MSC.** Gene expression levels of chondrogenic markers. MSC cultured for 7 days in presence of chondrogenic factors and HP stimulation. *p<0.05, **p<0.01, ****p<0.0001.
The suppression of chondrogenesis following the application of HP to the soft hydrogels correlated with a reduction in the nuclear levels of SMAD 2/3 (Figure 6-3a) and a minor reduction in NCAD expression (Figure 6-3b). An opposite effect was observed in the stiffer hydrogels, where the application of HP was observed to increase nuclear SMAD 2/3 levels (Figure 6-3a) and total NCAD (Figure 6-3b) secretion, although the overall intensity of staining for the proteins was lower in the stiff hydrogels compared to the soft hydrogels.
Figure 6-3 | The influence of HP on nuclear SMAD 2/3 and NCAD levels on MSC in soft and stiff hydrogels. Confocal analysis of MSC cultured for 7 days in presence of TGF-β3 and HP. Cells stained for nuclei (blue), actin (magenta) and a) SMAD 2/3 (grey) or b) NCAD (cyan). Scale bars, 10 μm. Graphs reporting the fold change of a) the nuclear content of SMAD 2/3 and b) the total expression of NCAD. *p<0.05, **p<0.01, ****p<0.0001.
6.3.2 Early HP stimulation suppresses MSC condensation and leads to epigenetic changes associated with osteogenesis

To understand the mechanism of HP mechanotransduction, this thesis next sought to analyse cytoskeletal and epigenetic changes in MSC cultured in soft and stiff hydrogel environments and exposed to TGF-β3 and HP. The intermediate filament architecture, in particular the vimentin network of MSC, has been shown to remodel under the action of HP\textsuperscript{99,241}. Although assessed only qualitatively, vimentin formed a less interlaced network when exposed to HP and this correlated with a decreased cell sphericity (Figure 6-4a). Surprisingly, the volume of cells seeded into a soft 3D IPN reduced significantly under HP stimulation, while such changes in cell size were not observed in the stiffer hydrogels (Figure 6-4a). In Chapter 5, this thesis described the tendency of MSC to form aggregates when seeded into soft 3D IPN. Following the application of HP, cell aggregation in the soft matrix was impaired, while no evident effect on cellular aggregation was observed in the stiffer 3D IPN (Figure 6-4b). Although HP didn’t affect significantly the tendency of cells to aggregate/condense in the stiffer 3D IPN, the average distance between single cells increased in both soft and stiff hydrogels after HP stimulation (Figure 6-4b).
Figure 6-4 | HP stimulation inhibits MSC aggregation. a) Confocal analysis of MSC cultured for 7 days in presence of TGF-β3 and HP. Cells stained for nuclei (blue), actin (magenta) and vimentin (green). Scale bar, 10 μm. Graphs reporting the values of sphericity and volume of single cells embedded into 3D IPN. b) Average percentage of cell aggregates per field of view after 7 days of culture and cell-cell distance. *p<0.05, **p<0.01, ****p<0.0001.

To further investigate how HP impaired MSC chondrogenesis, the expression of key osteogenic and hypertrophic markers was next studied. HDAC4 is known to be a potent regulator of chondrocyte hypertrophy by inhibiting RUNX2
expression\textsuperscript{26,242}. Nuclear HDAC4 levels were higher in soft gels compared to stiff gels. The application of HP was observed to reduce HDAC4 nuclear localization in MSC encapsulated in both soft and stiff hydrogels (Figure 6-5a). Furthermore, the application of HP enhanced the expression of $RUNX2$ and $COL1$ in the soft hydrogels, while no significant effect was observed in the stiffer gels (Figure 6-5b).
Figure 6-5 | HP induces epigenetic changes. a) Evaluation of HDAC4 localization and nuclear content. Cells stained for nuclei (blue), actin (magenta) and HDAC4 (yellow). Scale bar, 10 μm. b) Day 7 gene expression. *p<0.05, **p<0.01, ***p<0.001.

6.3.3 A delayed exposure to HP enhances chondrogenesis of MSC

To assess whether a delayed exposure to HP would enhance the chondrogenic differentiation of MSC, cells were cultured for 1 week in free swelling chondrogenic conditions, followed by a week of HP stimulation in the same media
conditions. In this case, the application of HP had no (positive or detrimental) effect on the expression of SOX9, ACAN and COL2 in MSC encapsulated within the soft 3D IPN, although it did suppress the expression of COLX. In contrast, HP enhanced the expression of SOX9, ACAN and COL2 in MSC encapsulated within the stiffer matrix which was previously found to be less supportive of a chondrogenic phenotype (Figure 6-6a). From a morphological standpoint, HP promoted cell volume expansion in the softer 3D IPN, but not in the stiffer hydrogel. The average distance between cells decreased with the application of delayed HP irrespective of substrate stiffness (Figure 6-6b). Surprisingly, an increase in cell aggregation with the application of delayed HP was only observed in the softer matrix (Figure 6-6c).
Figure 6-6 | Beneficial effects of delayed exposure to HP. a) Gene expression analysis of MSC cultured for 1 week in presence of TGF-β3 followed by 1 week of HP and TGF-β3. b) Graphs reporting single cell volume and cell-cell distance after 14 days of culture. c) Representative images of cell aggregates and their quantification. Cells stained for actin (magenta). Scale bar 100 μm. *p<0.05, **p<0.01, ****p<0.0001.
6.4 Discussion

It has been previously demonstrated by our lab that physiological levels of cyclic HP stimulation have beneficial effects on the long term chondrogenic commitment of bone marrow derived MSC\textsuperscript{96,97,243}. However, the mechanism by which the interplay of matrix stiffness, growth factors and HP influences MSC commitment has been less investigated. This study confirms that a biomaterial able to facilitate cell-cell interactions and aggregation over time creates a supportive environment for MSC chondrogenesis, and that applying cyclic HP after the initiation of chondrogenesis can further enhance and aid cell lineage commitment. In contrast to earlier studies\textsuperscript{93}, HP stimuli in absence of chondrogenic factors showed to be insufficient to promote chondrogenesis of MSC. Further, the combination of HP and TGF-\(\beta\) during the early stages of MSC differentiation inhibited chondrogenesis and promoted epigenetic changes associated with osteogenesis. In these studies, HP stimulation was associated with vimentin remodeling, cell volume restriction and a reduced degree of cell condensation. In contrast, when MSC were allowed to first initiate chondrogenic differentiation in free swelling conditions (i.e. one week of TGF-\(\beta 3\) supplementation), the application of HP no longer suppressed chondrogenesis. Indeed, such a delayed mechanical stimulation was found to enhance cell aggregation and suppress markers of hypertrophy in the soft 3D IPN, and enhance the expression of chondrogenic genes in the stiff hydrogels. These findings highlight that cyclic HP can directly modulate MSC fate in a manner that depends on substrate stiffness, growth factor stimulation and the timing of HP exposure. Altogether these studies provide a novel platform for MSC differentiation analysis and may open new possibilities to develop loaded-assisted cartilage tissue engineering strategies.
While bone marrow derived MSC are clearly responsive to HP in this hydrogel system, in absence of exogenous TGF-β3 supplementation, this mechanical stimuli didn’t promote chondrogenic lineage commitment. Indeed, mechanically loaded MSC grown for 7 days in the soft 3D IPN expressed lower levels of *ACAN*, while cells kept in the stiffer 3D IPN reduced their expression of *COL2* and upregulated *COL1*, perhaps indicative of a switch toward a more fibroblastic or osteoblastic phenotype. Previous studies showed that in absence of TGF-β, a loading regime of 10 MPa, 1Hz for 4hours per day increased the expression of *SOX9, COL2* and *ACAN* and matrix deposition after MSC were loaded for 14 days\(^9\). Further, a study employing a regime of 7.5 MPa described the upregulation of *SOX9* and *ACAN*, however despite this initial upregulation (after 1 week), no chondrogenic gene expression was detected at later time point due to loss of cell viability\(^244\). Similar to the observations reported in this thesis, a pellet culture without TGF-β supplementation and a loading regime of 0-5 MPa at 0.5 Hz for 4 hours/day for 10 days showed no chondroinductive effect of HP\(^245\). Therefore the role of HP in initiating chondrogenesis in the absence of soluble factors remains controversial, with the results reported herein pointing to a negative chondroinductive role of HP in absence of TGF-β.

The early application of HP loading, combined with TGF-β3 supplementation, was associated with a downregulation in chondrogenic markers within the soft 3D IPN. This was associated with a reduction in the nuclear levels of SMAD 2/3 and a reduced secretion of NCAD, suggesting a negative influence of HP stimulation. *In vitro*, MSC chondrogenic differentiation is elicited by cell condensation, which is mediated by NCAD\(^45\). However, as cells become chondrogenic, the expression of NCAD decreases\(^45\). Cells undergoing thorough condensation activate SMAD2/3 complex, which translocates in the nuclear
compartment where it regulates SOX9 expression, which in turn controls the transcription of the major cartilage matrix proteins COL2 and ACAN\textsuperscript{246}. In this study, the application of HP reduced the mRNA levels of ACAN, COL2 and COLX in MSC maintained in a soft matrix to levels comparable to that in a stiffer, unloaded, environment. The application of HP to the stiffer 3D IPN, which has been previously shown (see Chapter 5) to be a less chondroinductive environment, affected only the expression of CDH2, COLX and TGFBR2 genes. Conversely, HP was associated with an increase in SMAD 2/3 nuclear intensity and NCAD total production in these stiffer hydrogels, although the overall levels were still significantly inferior to MSC maintained a soft matrix in static or dynamic culture, hence still insufficient to trigger a more robust chondrogenic response.

Early HP loading was also associated with a decrease in cell aggregation in the softer 3D IPN, as well as vimentin remodelling, a decreased cellular volume and greater cell-cell distances. It is to be noted that the cell-cell distance has been calculated excluding cellular aggregates within the computational analysis. Being equal to 0 the distance of contacting cells, the estimation of the average cell-cell gap could have been reduced by including also cells within aggregates. From a morphological standpoint, the remodeling of the vimentin structure in cells kept in a soft 3D IPN wasn’t followed by a change in the overall cell sphericity, as it might have been expected from the cell volume restriction observed upon loading. Although reducing the extent of cellular aggregation, the early application of HP didn’t reduce the DNA levels within the hydrogels (see appendix V, Figure S-0-4).

Both matrix stiffness and HP were observed to play a role in vimentin remodelling and the morphology of the encapsulated MSC. The increase in matrix stiffness was correlated to vimentin remodelling and a decrease in cell sphericity, which wasn’t followed by cell volume restriction nor expansion. Such
morphological changes and the increase in the DNA content (see appendix V, Figure S-0-4) weren’t followed by an increase in cell aggregation, which can be associated with the increase in cell-cell distance observed after loading. It is know that vimentin architecture changes upon HP stimulation, both in 2D and 3D cell culture models\(^9,241\). It is hypothesized that vimentin might be more sensitive to HP and the pressurization could induce its depolymerisation\(^9,232\). In vivo, vimentin increases the cytoplasmic elasticity and it is responsible for the alignment of cell traction forces needed for directed mesenchymal migration; from rheology measurements, its network showed to be easily deformable and able to withstand high strains without breaking\(^247–249\). Although vimentin’s involvement with cellular response to HP stimuli, little is known about the correlation between vimentin adaptation and cell volume control. Cellular size control has been shown to be a robust modulator of MSC and chondrocytes fate. Indeed, MSC volume expansion was correlated to osteogenic differentiation, whilst chondrocyte confinement reduced the secretion of cartilage matrix protein, enhancing a more catabolic phenotype\(^176,181\). The results showed in this chapter might have shown for the first time a possible interplay between vimentin pressure-induced changes and cell volume adaptation and how this relationship can be modulated by the stiffness of a 3D matrix. Although being outside the aim of these studies, a deeper understanding of the interdependency of vimentin remodelling, cell volume adaptation and cell migratory behaviour would be of benefit in developing loaded-induced MSC differentiation strategies.

The nuclear localization of HDAC4 was found to depend on both substrate stiffness and hydrostatic pressure, suggesting epigenetic changes in MSC in response to such mechanical cues. It is known that mechanical perturbations can alter the state of the nucleus and in some cases the physical signals reach the nucleus
before the soluble ones\textsuperscript{250–252}. For instance, fluid flow induced shear stress modulated chromatin condensation and increased nuclear stiffness in endothelial cells\textsuperscript{253}. Twisting the cytoskeleton via magnetic beads, caused a direct force transmission to the nucleus and elicited local chromatin remodelling\textsuperscript{254}. Histone deacetylases (HDACs) participate in epigenetic regulation by keeping the chromatin in a highly packed form, wrapped around histones\textsuperscript{255}. In particular, HDAC4 is a potent regulator of chondrocytes hypertrophy and its nuclear transport is initiated by TGF-β through the activation of SMADs complexes\textsuperscript{26,256,257}. Subcellular relocation of HDAC4 can be modulated by physical signals, indeed chondrocytes compressive loading induced HDAC4 nuclear import and gene regulation\textsuperscript{258}. Although the role of HP on HDAC4 shuttling is not clear in the literature, these findings suggest that both a stiffer substrate and early HP exposure might inhibit the nuclear import of HDAC4. HP stimuli correlated with a reduced nuclear presence of HDAC4 in MSC kept into soft and stiff 3D IPN, although increases in \textit{RUNX2} and \textit{COL1} were only observed in the softer hydrogels, confirming the detrimental effect of HP at this stage of MSC chondrogenic differentiation. It is possible that MSC grown in the soft 3D IPN might be more sensitive to HDAC4 shuttling than cells experiencing a stiffer environment, which already demonstrated lower levels of nuclear HDAC4. Although these findings linked for the first time the role of substrate stiffness and HP as possible regulators of HDAC4 during MSC chondrogenic differentiation, more investigations would be needed to understand how these physical stimuli interact to control HDAC4 behaviour.

A delayed exposure to HP was associated with reduced cell-cell distances and increased cellular aggregation for MSC grown in soft hydrogels, and an upregulation of \textit{SOX9}, \textit{ACAN} and \textit{COL2} in cells encapsulated within stiffer
matrices. The delayed application of HP was also found to reduce the expression of \( COLX \) in MSC encapsulated within soft 3D hydrogels. At this stage of differentiation (day 14) a reduction of \( COLX \) may be indicative of this mechanical stimulus suppressing hypertrophy and terminal differentiation\(^{186}\). The dynamics of cellular condensation within these IPN hydrogels may play a key role in determining the temporal response of chondrogenically primed MSC to HP. It has been previously shown that MSC cultured in form of pellets positively responded to HP stimuli and their chondrogenic commitment was enhanced to a greater extent by TGF-\( \beta \) supplementation\(^{239}\). MSC cultured as pellets are forced to aggregate at the onset of chondrogenesis. In contrast, MSC encapsulated in hydrogels are initially relatively isolated, but in response to TGF-\( \beta \)3 stimulation begin to undergo chondrogenesis. As part of this process, and particularly in the soft IPN that support robust chondrogenesis, MSC began to form aggregates within the hydrogel. It is possible that early stimulation with HP might have suppressed MSC aggregation (especially for our low seeding density relative to other 3D hydrogel studies\(^{92,95,97,243,259}\)), whilst delaying HP to first allow MSC to condensate might have supported the more beneficial response to this mechanical stimulus that is typically reported in the literature. Interestingly, HP-stimulated cells maintained in soft matrices underwent volume expansion, while a reduction in cell volume was observed for MSC maintained in the stiffer hydrogels. Previous studies have associated volume expansion to an enhanced osteogenic differentiation of MSC\(^{176}\), while for chondrocytes the confinement of their volume inhibited cartilage matrix production\(^{73}\). In the previous chapter of this thesis, a similar tendency was observed, albeit cells volume underwent through a volume oscillation over time in culture. Although not directly examined, it is possible that volume regulation plays a key role...
role in the mechano-transduction of HP, however this hypothesis needs further examination.

To conclude, in presence of TGF-β, the application of 2MPa of HP for 4 hours per day from day 0 to day 7 generally inhibited cellular condensation and chondrogenesis. In contrast, the application of 4 hours of daily dynamic HP from day 7 to day 14 following TGF-β3 induced differentiation enhanced the chondrogenesis of MSC. Although the supplementation of supra-physiological level of TGF-β3 is a potent chondrogenic regulator, mechanical stimulation negatively modulate its effect if not applied at the correct timing, at least in part through cytoskeletal and epigenetic changes. It has yet to be established if the magnitude of the loading regime can influence the relationship between substrate stiffness and MSC response to TGF-β3, thus further analysis would be needed to establish the exact mechanism of mechanotransduction behind MSC response to HP. However, in the context of cartilage regeneration, this study demonstrated that physical stimuli are fundamental regulators of MSC fate determination, opening new avenues to loading-inspired strategies for cartilage tissue engineering.
Chapter 7  Discussion

The studies presented in this dissertation aimed to investigate how joint-specific *in vivo* environmental cues can be replicated and manipulated *in vitro* to influence the chondrogenic differentiation of MSC. The embryological development of cartilage tissue is characterized by a complex interplay of biochemical and biophysical cues. Cartilage morphogenesis is initiated by mesenchymal cells condensation in the early limb bud, which forms the cartilaginous anlage that gives origin to endochondral bone formation. Mechanical forces and local morphogen gradients greatly influence this process, hence biomimetic cartilage tissue engineering approaches, that aim to recapitulate the complex interplay of biophysical and biochemical cues, would open new possibilities for cartilage tissue regeneration\textsuperscript{6,260}. A reverse tissue engineering approach, exploiting knowledges from the mechanotransduction mechanisms of cartilage development, requires *in vitro* platforms to unravel the complex behavior of cell fate determination. Therefore, this thesis aimed to develop a biomaterial (IPN) able to support 2D and 3D cell growth, where both substrate stiffness and cell morphology could be independently tuned. Such a biomaterial allowed the examination and the direct comparison of MSC chondrogenic response in a 2D and 3D context. The pro-chondrogenic properties of a soft environment were demonstrated in both environmental contexts, as that softer matrix was correlated with enhanced upregulation of key chondrogenic markers and cell condensation. Conversely to the initial expectations, tuning the morphology of MSC in the 3D IPN to an initially spread shape, enhanced chondrogenesis compared to forcing the cell to initially
adopt a rounded shape. Once the correct combination of matrix rigidity and cell morphology that best supported chondrogenesis was identified, the final studies of this thesis examined the effect of hydrostatic pressure (HP) on the chondrogenic fate. In this case, physiological levels of HP applied in absence of chondrogenic factors or before cell condensation occurred had a detrimental effect on MSC chondrogenic commitment. In contrast, delaying the application of HP to allow MSC to first begin to aggregate and initiate chondrogenesis was found to enhance chondrogenesis.

The importance of cell condensation for MSC chondrogenesis has been explored through the different chapters of this thesis, however this phenomenon was first observed in response to the rigidity of the 2D IPN substrates. When stimulated with the chondrogenic growth factor TGF-β3, MSC seeded at a higher density on a soft 2D IPN were observed to assemble a smaller number of focal adhesions which was reflected on their morphology, as MSC maintained on this soft substrate were ultimately shown to be less spread than cells kept on a stiffer IPN. Cell condensation seemed favored by an initial higher cell seeding density and by a soft substrate, as confirmed by NCAD staining and the formation of larger cell aggregates/colonies. Chondrogenesis in this environment was supported by the nuclear translocation of SMAD 2/3 and by the upregulation of key chondrogenic genes. Notably, when MSC were cultured on a stiffer matrix under chondrogenic supplementation, YAP nuclear translocation was less evident, contrary to its mRNA expression levels and downstream activity. Similarly to these findings, Foyt et al. showed that a soft substrate (0.167 kPa) was able to induce MSC aggregation and chondrogenic commitment, which was associated with cytoskeletal tension at 2% O₂. Shifting from an oxygen tension of 20% to 2% allowed the formation of larger cell colonies on soft substrates, which were found to be 5 times smaller than cell
aggregates/colonies on stiff (49.6 kPa) substrates at 2% O$_2$. The compactness of cells on soft substrates was supported by NCAM upregulation in response to a decreased oxygen availability, whereas neither substrate stiffness nor oxygen tension influenced NCAD mRNA levels. Conversely this thesis, where experiments were conducted at an oxygen tension of 5% O$_2$, showed that although cells on both 2D IPN environments were forming a similar number of colonies, only the soft 2D IPN allowed the formation of colonies with larger size. Further, NCAD levels were found to be significantly higher at both gene and protein level on cells grown on soft 2D IPN and at either low or high seeding densities. Both studies agree on dissociating this cellular aggregation to proliferative difference driven by substrate stiffness. One of the major differences among the two studies regarded the findings on YAP behavior. As previously stated, YAP is considered to be a cellular rheostat, which can be physically activated (nuclear translocation) by substrate stiffness and cell morphology$^{24,124}$. Foyt et al.$^{164}$ observed lower nuclear levels of YAP on cells undergoing chondrogenic differentiation on soft polyacrylamide (PA) substrates, contrary to what one might expect with associating the chondrogenic enhancement to increases in cytoskeletal tension in 2D. The findings in this thesis reported a different finding, with YAP found to be present in the nucleus of MSC kept on the soft 2D IPN. This difference might be due to the range of stiffness on which the cells were grown (soft 2D IPN= 2.8 kPa against 0.167kPa of PA substrates) in combination to a different integrin engagement in response to either the collagen I of the 2D IPN or the fibronectin coating of the PA substrates. Although a head-to-head examination of YAP behavior would need the decoupling of the differences among the two studies, further analysis on the crosstalk among the Hippo and TGF-$\beta$ pathway might shed light on YAP’s involvement in MSC chondrogenic commitment and its modulatory role on SMAD2/3 cellular sublocalization$^{141}$.
The chondro-supportive ability of a soft environment was next assessed by the direct comparison of MSC lineage commitment in the 2D and 3D IPN. In both bases, a more compliant environment better supported chondrogenesis of MSC. In a 3D environment, MSC adopting a spread morphology could form more aggregates and expressed higher levels of key chondrogenic markers at the gene and protein level. Such chondrogenic differentiation was associated with enhanced SMAD 2/3 nuclear localization and lower levels of nuclear YAP compared to other hydrogel environments. In contrast, hindering cell spreading by forcing MSC to initially maintain a rounded morphology and a lower cell volume in the softer hydrogels led to increased expression of osteogenic markers. Therefore, this study demonstrated that a 3D IPN that provides a matrix stiffness\textsuperscript{163}, and supports cell morphologies\textsuperscript{36}, comparable to that of the developing cartilage anlage, enhances the chondrogenic potential of MSC, at least in part by supporting volume expansion and cellular aggregation. The differences reported between cells grown on the 2D IPN and into the 3D IPN might be explained by the robust influence of dimensionality on cell behavior. One example, cells on 2D surfaces can freely spread, migrate and rearrange, whereas in a 3D matrix cells need to squeeze through pores or degrade the matrix to migrate. Further, such constraints support the ambiguous assumption that increasing crosslinking of a matrix could enhance cell spreading and motility, while locking cells in such a 3D matrix is likely to retard cell motility and spreading\textsuperscript{261}. Describing dimensionality as a single variable would be a limited assumption, as cells can sense it on multiple levels. Further investigations to understand how dimensionality affect cellular behaviors would advance the development of biomaterials for tissue engineering application.

Harnessing the regenerative power of MSC for the repair of the musculoskeletal system requires the ability to mimic developmental pathways and
physiological conditions in vitro. The mechanical environment has an important influence on chondrogenesis, and in particular cyclic HP is known to be a key component of the mechanical environment of synovial joints. This thesis examined the interplay of physiological magnitudes of HP stimulation, substrate stiffness and TGF-β3 on the chondrogenic commitment of MSC. In absence of chondrogenic factors HP showed to be insufficient to support a chondrogenic genotype. The combination of HP and TGF-β3 during the initiation of MSC differentiation resulted in epigenetic changes associated with downregulation of chondrogenic markers and an enhancement of osteogenic genes in cells grown in a soft 3D IPN. These cells showed a remodeled vimentin network, cell volume restriction and a reduced degree of cell condensation. Allowing MSC to initiate their chondrogenic differentiation before HP stimulation resulted in a more beneficial response to the application of this mechanical stimulus. This delayed exposure to the mechanical perturbation enhanced cell aggregation in the soft 3D IPN and supported the chondrogenic commitment of cells kept in the stiffer environment. The role of HP is generally associated to a modulator of prehypertrophic and hypertrophic markers as confirmed by previous studies on adult MSC in a pellet culture system, following hydrogel encapsulation or in micromass culture of embryonic chondroci. Therefore, all together these findings might suggest that the timing of HP stimulation need to be finely tuned at later stages of MSC chondrogenesis in order to elicit its positive chondro-modulatory effect. Finally, this thesis demonstrated that it is possible to control MSC chondrogenic commitment, by modulating the synergism of substrate stiffness, growth factors and HP timing of exposure.
Taken together these findings showed a complex interplay of biophysical and biochemical stimuli, which need to be incorporated to informed next-generation biomaterials aimed to engineer cartilage tissue.
Chapter 8  Conclusions

The main goal of this thesis was to assess the optimal mechanical parameters able to elicit the chondrogenic differentiation of mesenchymal stem cells (MSC). The lineage commitment was analysed in a 2D and 3D hydrogel system (IPN), in which substrate rigidity and cell morphology could be independently tuned. An external mechanical stimulus, specifically hydrostatic pressure (HP), was applied in order to mimic the loaded environment of the knee joint and to investigate possible mechanism of mechanotransduction involved in MSC fate decision. The main results of this thesis are as follows:

- In 2D culture, the chondrogenic differentiation of MSC is associated with cell growth on a soft substrate. Such level of rigidity supports cell condensation and upregulation of key chondrogenic markers.
  - Higher seeding densities stimulate MSC proliferation, aggregation and enhanced chondrogenesis.
  - Changes in cell morphology and focal adhesion assembly are influenced by the density of MSC seeded onto a substrate.
  - Altered YAP signaling was observed during chondrogenesis of MSC. YAP nuclear localization increased on soft substrates.
  - MSC grown on soft 2D IPN form larger cell aggregates and undergo a more robust chondrogenesis.

- The embedding of cells into a more physiologically relevant environment, such as a 3D IPN hydrogel, greatly enhanced MSC chondrogenesis.
  - Dimensionality regulates key chondrogenic genes.
A spreading-permissive, soft 3D IPN allows cell aggregation, positive regulation of chondrogenic markers and cell volume expansion.

Hindering cell spreading is associated to inhibited chondrogenic initiation and an upregulation of YAP and osteogenic genes in soft 3D hydrogels.

The timing of the application of HP determines its influence on chondrogenesis of MSC.

- HP stimulation at the very onset of chondrogenesis reduces cellular aggregation, cell volume and the expression of key chondrogenic genes
- HP loading after 1 week of TGFβ mediated chondrogenesis enhances cell aggregation in soft 3D IPN and enhances the expression of a number of key chondrogenic genes in the stiff 3D IPN.

This thesis demonstrates that the initiation of MSC chondrogenesis can be mechanoregulated. The design of biomimetic biomaterials combined with bioreactor systems can lead to new tissue engineering strategies. Additionally, this work contributes to the general understanding of MSC mechanobiology and can be of benefit for many cell-based cartilage regeneration strategies.
8.1 Future Directions

Although this dissertation has brought significant insight into the mechanobiology of MSC, many questions remain to be answered. For this reason, the following suggestions are made available for future studies on the subject of this thesis:

- MSC aggregation on top of 2D IPN and into 3D IPN might be due to an enhanced migratory speed of the cells through the matrix. A time lapse analysis of cellular movement and/or traction force microscopy (TFM) analysis might be necessary to explore whether cell aggregation in soft 2D IPN and 3D IPN is due to a faster migratory speed. Further, knowledge of the level of cell-generated traction forces might reveal differences induced by dimensionality and might suggest different thresholds for MSC chondrogenic commitment initiation.

- To confirm the role of NCAD and cellular aggregation in the initiation of chondrogenesis seen in the IPN system, a mechanistic approach to target NCAD might be beneficial. The inhibition of NCAD might mimic the detrimental effect of stiffness on the chondrogenic fate of MSC.

- Although this thesis used physiological levels of HP, a systematic study on loading regimes might give further insight on the best HP parameters to use for loaded-induced strategies of MSC differentiation for cartilage regeneration.

- Finally, it has been shown that HP might cause epigenetic changes through HDAC4 translocation. A screen of possible miRNA or drugs that can mimic/inhibit the effect of HP, might add significant insight for cartilage regeneration in the context of cell based therapies.
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Appendices

Appendix I: MSC tripotentiality assessment

**Figure S-0-1 | MSC tripotentiality assessment.** Donor D42, cell stained for adipogenesis (Oil Red O, a), osteogenesis (Alizarin Red, b) and chondrogenesis (Alcian Blue, c) after 21 days of culture. Scale bars: 50 µm (a and b) and 1 mm (c).
Appendix II: Macro code for FIJI and Image J

```java
imageTitle = getTitle();
imageRoot = File.directory;
print(imageRoot);
path_for_results = imageRoot;

Channels = newArray("DAPI", "COL2", "SMAD23", "F-ACTIN");
ActiveChannels = newArray("ON", "OFF", "ON", "ON");
AutoThresholdMethod1 = newArray("Otsu", "Otsu", "Otsu", "Otsu");
AutoThresholdMethod2 = newArray("Otsu", "Otsu", "Otsu", "Otsu");
VolBoundingLimit = newArray(1000, 1000, 2000, 2000);

getBoolean("All Channel Names, threshold methods, threshold limits, and minimum bounding box values are set in lines 7 to 11 of the macro.

Results are 3D ROIs and are saved as a .zip file at "+path_for_results+". 
Click Cancel if details are not correct, and change in macro code.
Otherwise Click Yes")

// splits the channels
run("Split Channels");

// counts the number of open channels
numchannels = getList("image.titles");
```
for (i = 1; i <= numchannels.length; i++) {
    if (ActiveChannels[i-1] == "ON") {
        selectWindow("C"+i+-"+imageTitle);
        run("Gaussian Blur 3D...", "x=2 y=2 z=2");
        setAutoThreshold(AutoThresholdMethod1[i-1]);
        setOption("BlackBackground", false);
        run("Convert to Mask", "method="+AutoThresholdMethod1[i-1]+" background=Dark calculate black");
        run("3D Manager");
        Ext.Manager3D_Segment(128, 255);
        Ext.Manager3D_AddImage();
        Ext.Manager3D_Count(nROIs);
        if (i==1)
            Volume_cut_off = VolBoundingLimit[0];
        else if (i==2)
            Volume_cut_off = VolBoundingLimit[1];
        else if (i==3)
            Volume_cut_off = VolBoundingLimit[2];
        else if (i==4)
            Volume_cut_off = VolBoundingLimit[3];
        print("n"+Channels[i-1]+" All Objects:");
        for (j = 0; j < nROIs; j++) {
            
        }
Ext.Manager3D_Bounding3D(j,x0,x1,y0,y1,z0,z1);
VolBB = (x1-x0+1)*(y1-y0+1)*(z1-z0+1);
print("Object "+j+"\t Volbounding "+VolBB);
}

print("n"+Channels[i-1])+" Objects above "+VolBoundingLimit[i-1]);
for (k = 0; k < nROIs; k++)
{
Ext.Manager3D_Bounding3D(k,x0,x1,y0,y1,z0,z1);
VolBB = (x1-x0+1)*(y1-y0+1)*(z1-z0+1);
if(VolBB < Volume_cut_off)
{
Ext.Manager3D_Select(k);
Ext.Manager3D_Delete();
k=k-1;
nROIs=nROIs-1;
}
else
{
print("Object "+k+" Volbounding "+VolBB);
}
}
if(ActiveChannels[i-1]="ON")
{
    if (i==1)
        Ext.Manager3D_Save(path_for_results+imageTitle+
"+Channels[0]+.zip");
    else if (i==2)
        Ext.Manager3D_Save(path_for_results+imageTitle+
"+Channels[1]+.zip");
    else if (i==3)
        Ext.Manager3D_Save(path_for_results+imageTitle+
"+Channels[2]+.zip");
    else if (i==4)
        Ext.Manager3D_Save(path_for_results+imageTitle+
"+Channels[3]+.zip");
        Ext.Manager3D_Delete();
        Ext.Manager3D_Close();
}
}
close("*");

open(imageRoot + imageTitle);
run("Split Channels");
for (l=1; l <= numchannels.length; l++){

    if(ActiveChannels[l-1] == "ON")
    {
        selectWindow("C"+l+"-"+imageTitle);
        showMessage("i = "+l+" Channel is "+Channels[l-1]);
        setAutoThreshold(AutoThresholdMethod2[l-1]);
        setOption("BlackBackground", false);
        run("Convert to Mask", "method="+AutoThresholdMethod2[l-1]+" background=Dark calculate");
        rename(imageTitle+" "+Channels[l-1]);
    }
    else{
        selectWindow("C"+l+"-"+imageTitle);
        close();
    }
}

run("3D Manager");
run("Tile");
Appendix III: comparison of mechanical properties of 2D and 3D IPN

Figure S-0-2 | Mechanical properties of 2D and 3D IPN.
Appendix IV: DNA quantification in 3D IPN

Samples (n ≥ 4) were washed in PBS, the wet mass recorded and stored at -80°C. Samples were mechanically digested by means of disposable pestles and then enzymatically digested with papain (125μg/ml) in 0.1 M sodium acetate, 5mM L-cysteine-HCl and 0.05 M EDTA (pH 6.0, all Merck) at 60°C under constant rotation for 18 hours followed by the addition of 1 M sodium citrate solution for 1 h to dissociate crosslinked alginate. DNA content was determined using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermoscientific) following the manufacturer protocol, normalized to the respective dry weight and normalized to day 0 samples.

Figure S-0-3 | Biochemical analysis of DNA content. MSC grown for 7 days in 3D IPN show same levels of DNA when compared to day 0 (dotted line).
Appendix V: effect of HP stimulation on MSC proliferation.

Immediately after loading, samples (n ≥ 4) were wased in PBS, the wet mass recorded and stored at -80°C. The samples were mechanically and then enzymatically digested in a papain solution ((125μg/ml) in 0.1 M sodium acetate, 5mM L-cysteine-HCl and 0.05 M EDTA pH 6.0, all Merck) ) at 60°C under constant rotation for 18 hours followed by the addition of 1 M sodium citrate solution for 1 h to dissociate crosslinked alginate. DNA level was detected with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermoscientific) following the manufacturer protocol and the results normalized to the dry weight were reported as fold change of day 0 values.

Figure S-0-4 | Effect of HP on DNA content. Analysis of the DNA content of MSC cultured for 7 days in presence of TGF-β and HP. Values reported as fold change of day 0, indicated by the dotted line. * p<0.05, **p<0.01, & reports p<0.05 respective of day 0 values.