ChondroGELesis: hydrogels to harness the chondrogenic potential of stem cells

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Abstract

The extracellular matrix is a highly complex microenvironment, whose various components converge to regulate cell fate. Hydrogels, as water-swollen polymer networks composed by synthetic or natural materials, are ideal candidates to create biologically active substrates that mimic these matrices and target cell behaviour for a desired tissue engineering application. Indeed, the ability to tune their mechanical, structural, and biochemical properties provides a framework to recapitulate native tissues. This review explores how hydrogels have been engineered to harness the chondrogenic response of stem cells for the repair of damaged cartilage tissue. The signalling processes involved in hydrogel-driven chondrogenesis are also discussed, identifying critical pathways that should be taken into account during hydrogel design.

Keywords: hydrogel, chondrogenesis, mesenchymal stem cell

Contents

1. Introduction

Articular cartilage is found in diarthrodial joints and is responsible for load bearing and lubrication. These properties are essential for joint function and can be severely disrupted following damage to the cartilage, via physical trauma or disease, which results in a loss of protection against joint friction that leads to stiffness and pain in patients. A major challenge in joint therapy is that cartilage tissue cannot be regenerated by resident cells, known as chondrocytes, due to a lack of vascularisation in the tissue (1). Autologous transplantation of healthy cartilage and subchondral drilling are examples of current treatments that have limited availability and poor regenerative capacity (2). Thus, there is a significant drive to develop novel treatments for cartilage repair, particularly using biomaterials and stem cells to engineer healthy cartilage tissue.

Indeed, stem cells are capable of specialising into different cell types and of forming whole tissues. While there are many known types of stem cells in the body, embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs) are the most utilised in regenerative medicine research (3). MSCs are of particular interest in musculoskeletal rehabilitation as they can differentiate into myocytes, chondrocytes, adipocytes and osteoblasts and are utilised to engineer cartilage, bone, fat and muscle tissues among others (4). In cartilage tissue engineering, other more specialised cell types, such as human articular chondrocytes (ACs) and chondroprogenitor cells, have also been utilised. However, cell sourcing and issues with cell expansion limit their regenerative capacity (5).

In tissue engineering, the fate of the chosen cell type is usually directed by a biocompatible scaffold that hosts the cells and provides them with an extracellular matrix (ECM)-mimetic environment. Indeed, besides supporting cell growth, differentiation, and ECM remodelling (6), scaffolds can provide biochemical and physical cues, such as elasticity (7), that direct stem cells to differentiate towards certain lineages (8). Scaffolds that mimic the natural ECM of cartilage in a structural, mechanical, and biochemical manner can hence provide a suitable niche to promote cell adhesion, proliferation and chondrogenic differentiation. In the context of stem cell chondrogenesis, hydrogels represent a highly desirable scaffold candidate, due to their ability to mimic structural, mechanical and biochemical properties of the native ECM. These water-swollen polymer networks can be synthesised with desirable characteristics to promote chondrogenesis (9, 10). Furthermore, they provide a platform for injectable scaffolds (11), reducing the need for more invasive surgical treatments. Crucially, hydrogels allow stem cell encapsulation within a three-dimensional (3D) environment; this is essential to achieve a suitable chondrogenic niche. Indeed, studies addressing dimensionality in chondrogenesis have shown that a 3D culture improves chondrogenicity and reduces the hypertrophic and fibrocartilage phenotype compared to a two-dimensional (2D) environment (12, 13). It is also known that mesenchymal condensation is a crucial process for cartilage development during embryogenesis; a highly dense 3D cellular environment can facilitate this process through enhanced cell-cell contact and communication (14, 15). Hence, most of the

studies that have used hydrogels to harness stem cell chondrogenesis have been conducted in 3D.

In this review, we present and discuss recent developments in the design of hydrogels for stem cell chondrogenesis (**Figure 1**). Specifically, we look at how tuning the mechanical, structural, and biochemical (including release of chondroinductive factors) properties of the hydrogels can be used to direct stem cell fate towards a chondrogenic phenotype. We also consider the role of the cellular component of the microenvironment and of external cues applied to cell-laden gels. Finally, we examine the key signalling pathways and events that have been implicated in the chondrogenic response of stem cells via hydrogel engineering.

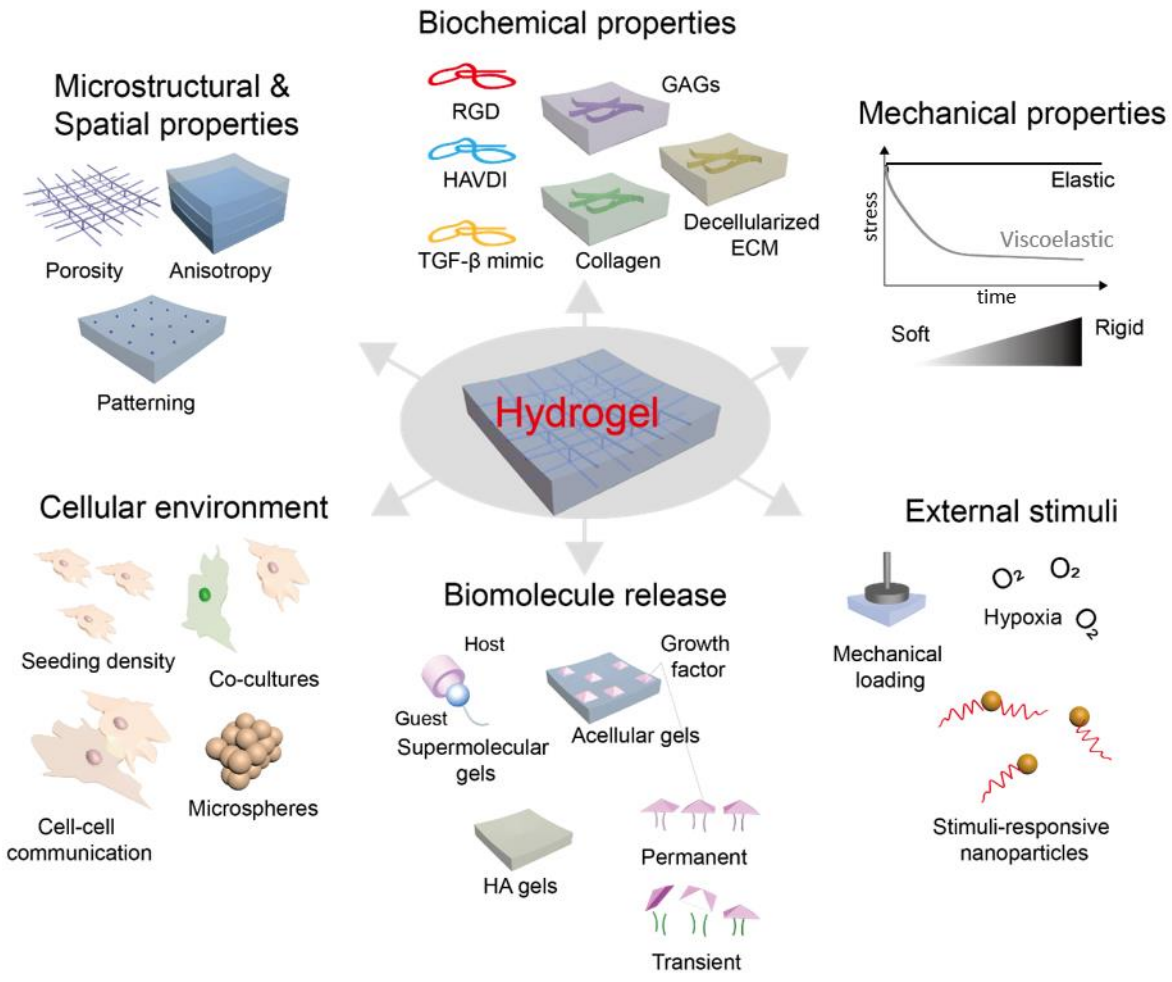

Figure 1. Properties and cues of the hydrogel microenvironment that regulate stem cell chondrogenesis.

2. Hydrogel composition

A broad variety of materials have been developed and utilised to fabricate hydrogel scaffolds for studies into stem cell chondrogenesis. These materials are selected to

fulfil specific criteria, including biocompatibility, biodegradability and sufficient mechanical strength to support tissue formation. Generally, they can be naturally derived or synthetic, with each type having their advantages and disadvantages (**Table 1**). Indeed, many studies have reported the development of hybrid hydrogels, which combine the advantages of various types of materials (**Figure 2**).

2.1. Natural materials

Naturally-derived hydrogels are often fabricated from animal- or microbial-derived polypeptides or polysaccharides. They have the advantage of being native to tissues and as such they can be good ECM mimics. Moreover, they often provide sufficient physical and biochemical cues to promote cell adhesion and differentiation (9).

Collagen-based hydrogels are one of the most prominent materials used to promote chondrogenesis (16-19), particularly using type I over type II collagen because it can facilitate chondrogenesis (20, 21) and more effectively represses inflammation and adverse immune-derived side effects (22, 23). Collagen is the most abundant component of the ECM, making it a popular choice in the fabrication of hydrogels. Furthermore, collagen hydrogels can be developed with tuned mechanical and structural properties to promote chondrogenesis. While these hydrogels may provide good *in vitro* environments to promote chondrogenesis, they often promote inflammation when implanted *in vivo*, resulting in unwanted side effects and supressed tissue formation (18). Furthermore, weak mechanical strength often results in mechanical failure upon implantation, further reducing their efficacy at promoting chondrogenesis and subsequent cartilage formation.

An alternative material to collagen for developing hydrogels is gelatin, a denatured derivative of collagen (24, 25). Gelatin is thought to maintain the advantages of collagen-based hydrogels, such as presentation of adhesion peptides (26), but with reduced cost and low immunogenic response (24). Gelatin also shows thermoreversible properties: it is soluble in water above 40^{o}C, when the chains behave as random coils, while upon cooling a transition occurs causing a hydrogel to form. Indeed, extended physical cross-links are formed by partial reversion to ordered triple helical segments, separated by peptide residues still in the random coil configuration (27, 28). The thermoreversibility of gelatin allows for a precise control of its gelation; this makes it advantageous for bioprinting applications, for example in cartilage tissue engineering, allowing for efficient and tailored treatment of unique defect patterns (29). Gelatin can also be actively remodelled by MSCs; this is fundamental in tissue engineering, since remodelling facilitates the generation of site-appropriate, functional tissue following disease or trauma (30). Many studies have reported that gelatin degradability and susceptibility to remodelling, amongst other features, are ideal material properties for cartilage tissue engineering (9). It has also been shown that remodelling of gelatin by MSCs causes dynamic shifts in the matrix environment and alters their autocrine and paracrine signalling (31). Additionally, the inhibition of MSC matrix proteases has been found to favour a fibrocartilage phenotype over hyaline cartilage, highlighting the significance of matrix remodelling in regulating chondrogenic outcomes (32).

Polysaccharides are also popular materials for developing hydrogels to use in tissue engineering (33). One of the most used polysaccharides is alginate, which has good biocompatibility and low immunogenicity (4, 34). It has also been shown that hybrid polysaccharide gels containing alginate can enhance stem cell chondrogenesis, for example in combination with collagen (35) or with the sulphated seaweed polysaccharide fucoidan (36). However, alginate lacks cell adhesion sequences, meaning that it must be functionalised to promote cell adhesion, proliferation, and differentiation.

Glycosaminoglycans (GAGs) are another popular material used to manufacture chondroinductive hydrogels. Hyaluronic acid (HA) is the most widely used of these due to its highly bioactive nature (26, 37). It is a predominant ECM component of several tissues, including cartilage (38); this makes it an attractive material for chondrogenesis. Furthermore, the biophysical properties of HA hydrogels can be fine-tuned, e.g. by varying crosslinker to HA ratio. Like alginate, HA lacks naturally occurring cell adhesion sites and must be functionalised to allow cell adhesion. Furthermore, the crosslinkers used to alter the physical properties of the hydrogels may result in cell toxicity, limiting the type and concentration of crosslinker that can be used. Another commonly used GAG in cartilage tissue engineering is chondroitin sulfate (CS). CS is a naturally abundant structural component of cartilage that plays a significant role in its resistance to compression (39). It has also been shown to inhibit cell attachment to adhesive ECM proteins by directly interacting with them and masking their adhesive sites from cells (40-42). Therefore, caution must be exercised when using CS for hydrogel development in tissue engineering to ensure that adequate cell attachment can be achieved. Another popular GAG used in hydrogel development is heparan sulfate (HS), which has been shown to promote cell spreading and focal adhesion formation (43). GAGs influence stem cell chondrogenesis in different ways depending on the type and amount used within hydrogels. For instance, the addition of HA to form hybrid gels with other materials has been shown to increase stem cell chondrogenesis (26, 44-46). However, high HA content has been shown to lead to hypertrophic cartilage formation (47) and to inhibit MSC chondrogenesis (48). On the other hand, low/moderate amounts of HA were found to be optimal for CD44 binding (48), which is crucial for HA-induced chondrogenesis (49). Additionally, the chondrogenic effects of the different types of GAGs have been compared: CS was shown to be more chondroinductive than HS (50), and both HA and CS were found to support neocartilage formation compared to HS which led to fibrocartilage (51). The different adhesive and chondroinductive properties of these GAGs should therefore be carefully considered when developing hydrogels for cartilage engineering.

Animal-derived components and decellularised tissues have also recently been used to develop chondroinductive hydrogels. Indeed, several studies have shown that these natural-derived materials can facilitate stem cell chondrogenesis, either by using scaffolds composed by decellularized ECM alone (52-54), or by adding decellularized tissue and natural components, such as platelet-rich plasma (PRP), to other gels (as soluble cues or to form hybrid materials) (55-60). Decellularized tissues can retain the biochemical and physical cues, including mechanical strength, found in the cartilage microenvironment, providing a powerful platform for promoting chondrogenesis of

stem cells. However, immunogenic responses and loss of ECM cues following harsh decellularization processes still hinder their use.

Other less intensely investigated materials for chondroinductive hydrogel fabrication include fibrin, a material usually used as a natural adhesive in reconstructive surgeries (61), Ɣ-poly (glutamic acid) (62), and chitosan, a crustacean-derived polysaccharide that has been widely investigated as a biomaterial (63).

2.2. Synthetic materials

While most publications have focused on naturally derived materials to fabricate chondroinductive hydrogels, some researchers have chosen to use synthetic materials. These may have certain advantages over natural hydrogels, such as a more precise control over the biomechanical properties, reproducible fabrication and lack of immunogenic response (64). Furthermore, biochemical cues can be incorporated into synthetic gels and precisely regulated in concentration and localisation (65).

The most common synthetic material used for hydrogel fabrication in tissue engineering is polyethylene glycol (PEG). While PEG is a bioinert material, it has been widely used to promote tissue regeneration. This is because the physical and biochemical properties of PEG hydrogels can be fine-tuned with relative ease via changing polymer concentration, crosslinker density, or by incorporation of other materials and biological molecules. Hence, PEG has been used in many publications to promote chondrogenesis (2, 8, 50, 66, 67). For example, a recent paper reported the development of a PEG-based composite hydrogel, where the incorporation of poly-D,L-lactic acid (PDLLA) and graphene oxide improved its bioactivity and stiffness (67). Other authors instead incorporated pro-chondrogenic factors, such as CS and HS, to improve the bioactivity of the hydrogels (50). Despite desirable qualities such as mechanical strength, PEG-based hydrogels often require additional functionalisation to promote chondrogenesis, which is a disadvantage compared to many hydrogels derived from natural materials.

Besides PEG, other synthetic materials have been used to develop hydrogels for studies on stem cell chondrogenesis. Polyacrylamide (PA) is a popular material in general hydrogel research due to its readily tuneable mechanical properties and potential for biochemical modification. It has been used extensively in many fundamental 2D studies, particularly concerning mechanobiology, including how stiffness regulates MSC differentiation towards specific lineages (7). Lack of biodegradability and toxicity of precursor components are some of the main limitations of PA hydrogels that prevent their use in 3D cell culture (68). However, it has been shown that PA hydrogels can be modified appropriately to support stem cell chondrogenesis in 2D (69).

Poly(N-isopropylacrylamide) (PNIPAM) is a synthetic temperature-sensitive polymer that has been used in recent studies developing hydrogels for tissue engineering (70). A unique property of PNIPAM is its ability to expel its liquid contents at a defined temperature by undergoing a reversible phase transition from a hydrophilic to hydrophobic state (70). This transition temperature can be chemically tuned to reach body temperature (71), which makes it highly desirable as an injectable hydrogel

system with controllable swelling properties. However, despite showing good biocompatibility, limitations of PNIPAM hydrogels include brittle properties at room temperature, poor mechanical properties, low swelling ratio, and slow kinetics of the volume phase transition in response to stimuli (72-74). This means that they are often used in combination with other materials that can bolster mechanical properties whilst retaining temperature-sensitivity. Specifically, PNIPAM gels have been shown to promote stem cell chondrogenesis as composite materials (46, 75-77).

Self-assembling peptide gels have also been used as materials to support stem cell chondrogenesis (78-83). Peptide gels are biocompatible, biodegradable, and possess self-healing and shear-thinning properties which are desirable in injectable gels and for use as bioinks (84). However, bare peptide gels lack cell adhesion motifs and require functionalisation to permit cell attachment (84). Moreover, they have low mechanical strength and stability, which needs to be improved using chemical crosslinking techniques, which can be toxic for cells (85).

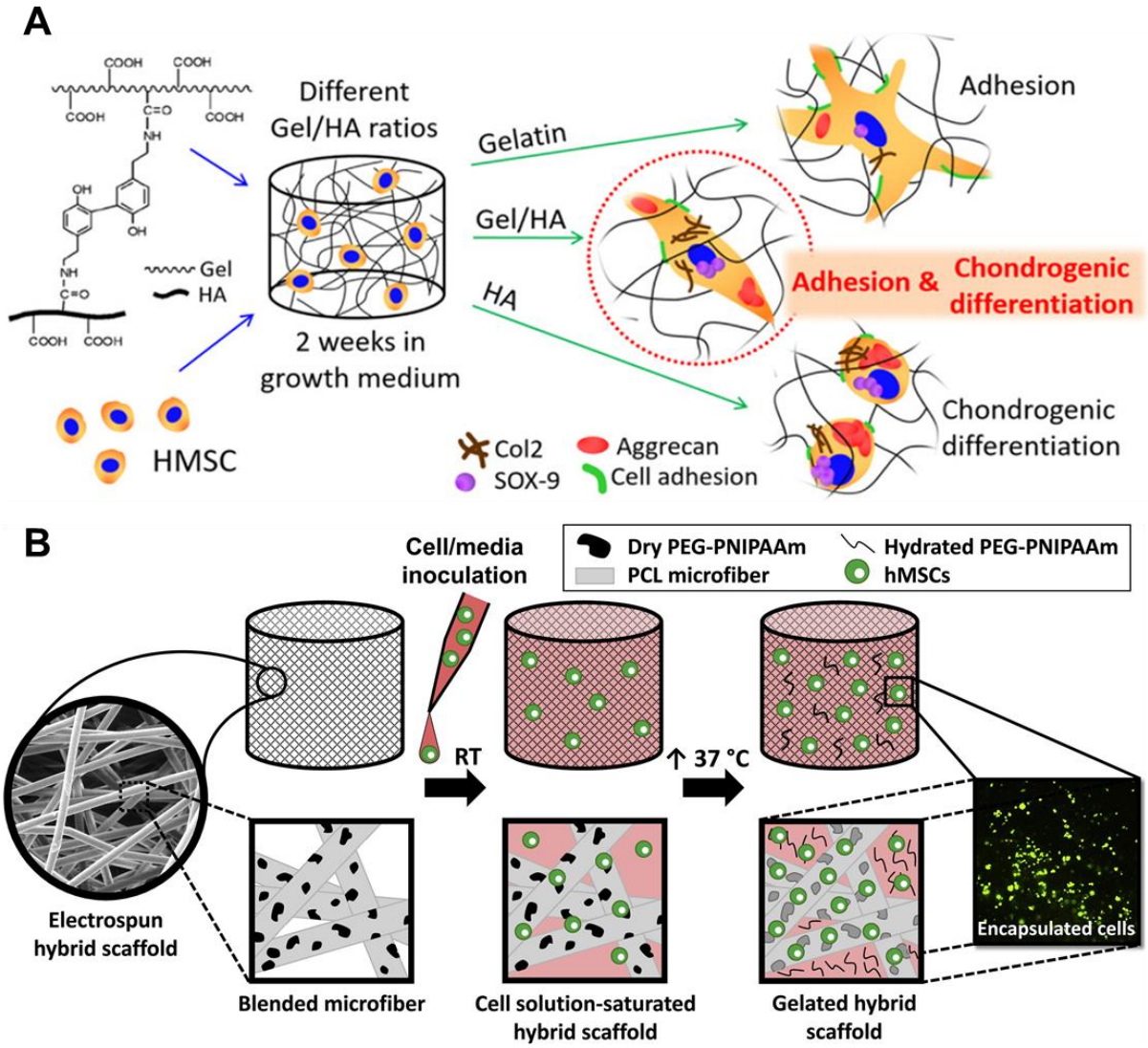

Figure 2. Hybrid gels that promote chondrogenesis. **A)** Optimised natural hybrid gels composed of 70% HA and 30% gelatin provide an environment that suitably balances cell adhesion and chondrogenic differentiation for potential use as an

injectable material for cartilage tissue engineering. Reproduced with permission (26). **B)** Hybrid synthetic scaffold harnesses the merits of various materials using the reverse thermosensitivity of PEG-PNIPAM and the mechanical support of PCL microfibers to effectively encapsulate cells in a 3D hydrogel with ideal viscoelastic properties. Reproduced with permission (75).

	Material	Advantages	Disadvantages	References
Natural	Collagen	Major ECM component, presentation of adhesion motifs, biocompatible, biodegradable	Mechanically weak, immunogenic response, expensive	(16-19)
	Gelatin	Presentation of adhesion motifs, low immunogenicity, biocompatible, biodegradable, thermoreversible, cost-effective	Mechanically weak	(24-28)
	Hyaluronic acid	Biocompatible, major component of cartilage	No cell adhesion sites, chemical crosslinking toxic to cells, excess inhibits chondrogenesis	(26, 44-46) (47, 48)
	Chondroitin sulfate	Biocompatible, major component of cartilage	Inhibits cell attachment to adhesive ECM proteins	(50) (51)
	Heparan sulfate	Biocompatible, promotes cell-cell and cell-matrix interactions	Leads to fibrocartilage	(50) (51)
	Alginate	Biocompatible, low immunogenicity	Lack of cell adhesion sites	(4, 34-36)
	Decellularized cartilage	Can retain mechanical, structural, and biochemical properties of native tissue	Harsh decellularization removes biochemical factors, immunogenic response	(52-60)
Synthetic	Polyethylene glycol	Tuneable degradability and mechanical properties, low immunogenicity, controlled presentation of biochemical cues	Requires functionalisation to promote cell adhesion and differentiation	(2, 8, 50, 66, 67)
	Polyacrylamide	Tuneable mechanical properties, controlled presentation of biochemical cues	Lack of degradability, toxicity of precursor components prevents 3D cell culture	(69)
	Poly(N-isopropylacrylamide)	Biocompatible, tuneable temperature-sensitive properties useful for injectable applications, controllable swelling properties	Brittle at room temperature, poor mechanical properties, low swelling ratio, slow kinetics of the volume phase transition in response to stimuli	(46, 75-77)
	Peptide gels	Biocompatible, biodegradable, self-healing, shear-thinning, use as injectable gels and bioinks	Requires functionalisation for cell attachment, poor mechanical strength and stability unless chemically crosslinked, toxicity of crosslinkers	(78-83)

Table 1. Commonly utilised natural and synthetic materials for hydrogel fabrication in the context of stem cell chondrogenesis.

2.3. Peptide-functionalised hydrogels

As anticipated in the previous sections, many hydrogels are composed of natural or synthetic polymers endowed with reactive moieties that can be readily modified with bioactive molecules. Numerous studies have decorated hydrogels with a single type of peptide, for example to allow cell adhesion to an otherwise non-adhesive polymer, or with combinations of biomolecules that could influence stem cell chondrogenesis. Using peptides that mimic the functional properties of full-length proteins often presents a more simplified and cost-effective alternative to using full-length proteins themselves, for instance with transforming growth factor-ß (TGF-ß) and CD44 (86). Hence, hydrogels modified with specific peptide cues can harness the chondrogenic potential of stem cells and hold great potential in cartilage engineering applications (**Table 2**).

To this end, functional peptide motifs of proteins with known chondroinductive properties, such as TGF-ß3, have been used. For example, the addition of aggrecan and TGF-ß3 mimic peptide SPPEPS to HA-based hydrogels was shown to enhance rat bone marrow-derived mesenchymal stem cells (BMSC) chondrogenesis through increased collagen II expression in 2D (87). Functionalisation of HA gels with another TGF-ß mimic peptide, cytomodulin-2, also enhanced the chondrogenesis of human periodontal ligament stem cells (PLSCs) *in vitro* and *in vivo* in mice (88) .

Modifying hydrogels with combinations of chondroinductive and degradable peptides has also been shown to collectively enhance chondrogenesis. Indeed, bacterial collagen-based hydrogels functionalised with matrix metalloprotease MMP7- and aggrecanase ADAMTS4-cleavable peptides and with chondroinductive heparin- and HA-binding peptide sequences were found to enhance the chondrogenesis of human MSCs (16).

The use of acellular gels modified with peptides have also been shown to effectively promote stem cell chondrogenesis *in vivo*. Modification of hybrid self-assembling peptides and decellularized porcine cartilage matrix hydrogels with bone marrow homing peptide PFS was in fact shown to enhance rabbit BMSC homing and chondrogenesis in 2D *in vitro* models and *in vivo* in rabbit (79). Acellular gel systems can be advantageous over cell-laden gels as they facilitate *in vivo* chondrogenesis solely through the biochemical cues of the hydrogels without the requirement for cell encapsulation, as further elucidated in section 2.4.2.

As well as using biomolecules with known chondroinductive properties, some of the most widely used peptides are those involved in fundamental cell processes that, in turn, can influence differentiation. These peptides are often functional domains/motifs of proteins, such as fibronectin and N-cadherin, that are involved in cell-matrix and cell-cell interactions respectively.

2.3.1. Fibronectin peptides

Fibronectin is a high molecular weight glycoprotein that is prevalent in the ECM and has been implicated in a variety of cellular processes, including growth, migration, and differentiation (89). The crucial function of fibronectin is cell adhesion, which is facilitated through binding to cell-presented integrins. Arginylglycylaspartic acid (RGD)

is the main adhesive peptide motif present in fibronectin, amongst other proteins, identified as the minimal recognition sequence for cell attachment (90). The functionalisation of hydrogels with RGD enables cells to physically engage with the microenvironment more effectively; this may improve their sensitivity to mechanical and biochemical matrix cues that influence differentiation.

Various studies have investigated the influence of RGD-functionalised hydrogels on stem cell chondrogenesis. Indeed, human posterior-derived progenitor MSC chondrogenesis was found to be enhanced on PEG-based hydrogels when RGD was present compared to gels without RGD (91). The combined effects of RGD functionalisation and other matrix cues have also been shown to collectively facilitate chondrogenesis. This has been for example investigated using hydrogels made of bacterial collagen-like protein Scl2. Scl2 has been used in many recent tissue engineering studies as an alternative to mammalian collagen, and can be functionalised with bioactive and biodegradable motifs, including GAG-binding peptides and biodegradable crosslinkers for MSCs chondrogenesis (92-100). In contrast to mammalian collagens, Scl2 shows minimal adverse immune response, low cytotoxicity, and can be recombinantly produced in high yields with minimal batch variation (101). Using Scl2 hydrogels, the role of the synergistic effect of cell adhesion and matrix remodelling in regulating chondrogenic fate has been investigated: indeed, MMP-cleavable RGD on Scl2 gels was found to enhance human MSC chondrogenesis compared to gel with permanently tethered RGD (83). Similarly, the interplay between adhesiveness and stiffness improved rabbit MSC chondrogenesis in PEG-gelatin-methacryloyl (GelMA) gels, with stiffer (25 kPa), higher RGD density (0.5%) gels performing better than 0.05% RGD density or softer (1.6 or 6 kPa) gels (102). The adhesive cross-talk between integrins and cadherins was also demonstrated to facilitate human MSC chondrogenesis, when norbornene-HA gels were functionalised with specific ratios of RGD and N-cadherin motif HAVDI (103). Moreover, the addition of RGD to HA-based gels further enhanced the chondroinductive effect of aggrecan and TGF-ß3 mimic peptide SPPEPS (87).

Collectively, these studies highlight how controlling cell adhesion to the hydrogels can improve the sensitivity of stem cells to other cues within the microenvironment, ultimately regulating chondrogenesis.

2.3.2. N-cadherin peptides

N-cadherin is part of the classic cadherin family of transmembrane glycoproteins that are important for mediating interactions between adjacent cells through a homophilic binding mechanism (104). Peptide sequences, such as HAVDI, derived from the homophilic binding site extracellular domain 1 (ECD1) (105), have been shown to be highly specific N-cadherin agonists (106). It is thought that using hydrogels modified with functional domains of N-cadherin can facilitate cell-cell communication, clustering, and condensation of MSCs, which are important events in chondrogenic differentiation and endochondral bone formation (107). Indeed, N-cadherin binding motif low-density lipoprotein receptor-related protein 5 (LRP5) enhanced mouse BMSC chondrogenesis when functionalised to alginate gels, particularly at low seeding densities (108).

HAVDI-functionalised hydrogels have been shown in various recent studies to significantly influence the chondrogenesis of stem cells. Using methacrylated HA gels, the presentation of HAVDI was demonstrated to enhance human MSC chondrogenesis in a dose-dependent manner; this was not observed when HAVDI was presented transiently, suggesting that persistent signalling is crucial for effective cell-cell-driven behaviour (38, 109). Studies using HAVDI-functionalised methacrylated HA gels have also shown that cadherin-mediated chondrogenesis is associated with ß-catenin signalling. Indeed, increased hMSC chondrogenesis was found to correlate with higher nuclear ß-catenin, particularly for instances where cell clustering occurred, highlighting the importance of cell-cell communication in chondrogenesis (109, 110). HAVDI-mediated β-catenin signalling has also been implicated in chondrogenesis using hydrogels formed by self-assembling peptide KLD-12. KLD-12 is composed of repeating alternative sequences of lysine (K), leucine (L), and aspartic acid (D), which form free-standing hydrogels due to hydrophobic and electrostatic interactions (111-115). The nanofibers formed by this peptide resemble fibrous cartilaginous extracellular matrix, providing a 3D microenvironment with biomimetic nanoscale architecture that supports the chondrogenic differentiation of hMSCs (116-119). When functionalised with HAVDI, KLD-12 gels were found to enhance chondrogenesis through inhibition of canonical Wnt/ß-catenin signalling, leading to more ß-catenin degradation (80). Additionally, rabbit MSC chondrogenesis was enhanced in a dose-dependent manner when hybrid poly(N-isopropylacrylamide-co-glycidyl methacrylate) (PNIPAM-co-GMA) gels were crosslinked with poly(glycolic acid) poly(ethylene glycol) poly(glycolic acid) di(but-2-yne 1,4 dithiol) (PdBT) that had been click-modified with HAVDI (127). PdBT is a highly versatile crosslinker that can be conjugated to a range of bioactive motifs, including HAVDI and CS, via simple mixing in water at room temperature (128); this makes it an attractive hydrogel crosslinker for tissue engineering applications.

Finally, hydrogels modified with combinations of HAVDI and other molecules have also been shown to be an effective strategy to enhance stem cell chondrogenesis. Indeed, as anticipated in section 2.3.1, specific ratios of HAVDI with adhesive peptide motif RGD in norbornene-HA gels facilitated human MSC chondrogenesis, indicating that tuned synergistic cell-cell and cell-matrix signalling can effectively modulate differentiation (**Figure 3**) (103).

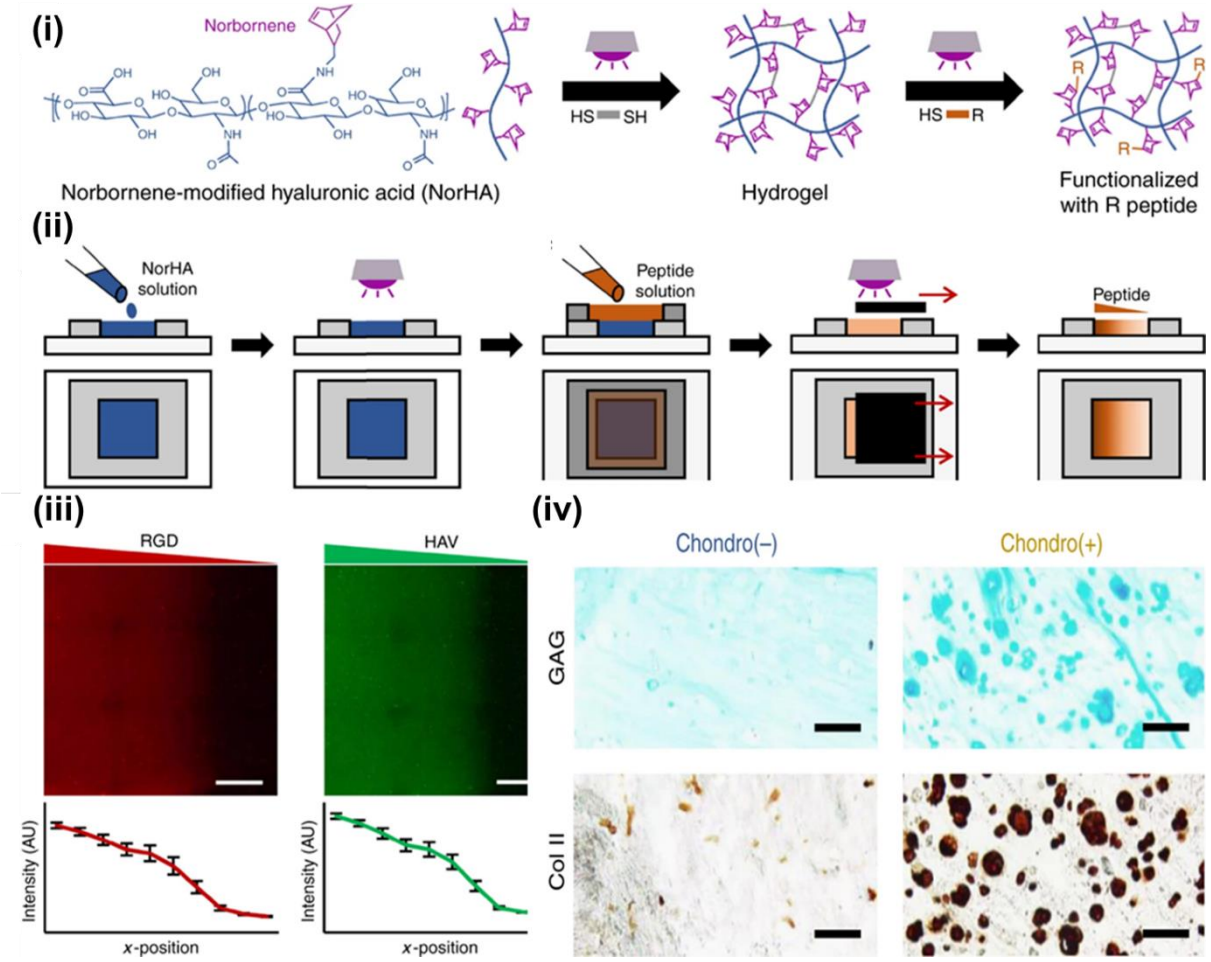

Figure 3. MSCs photoencapsulated in norbornene-modified HA hydrogels functionalized with peptide gradients (0–5 mM) of RGD and HAVDI that mimic cell-cell or cell-matrix interactions where optimal ratios of both facilitate chondrogenesis. i) Multi-step thiol-norbornene UV light-mediated reaction to form peptide-functionalised NorHA hydrogels. **ii)** Schematic of fabrication process involving NorHA hydrogel formation under UV light, incubation with mono-thiolated peptide solution, and introduction of peptide gradients introduced using an opaque sliding mask to control the extent of light-mediated reaction between peptides and norbornenes. **iii)** Images and quantification of signal intensity from gradients of fluorescently labelled RGD and HAV peptides. **iv)** Characterisation of chondrogenesis from gels with optimal RGD/HAV ratios by immunohistochemistry for GAGs and Col II after 56 days. Reproduced with permission (103).

Peptide	Gel type	Protein	Function	Cell type	Cell response	References
RGD	Scl2, HA, PEG	Fibronectin	Minimal recognition sequence for cell adhesion	Human posterior-derived progenitor MSCs, human MSCs, rabbit MSCs, rat BMSCs	Enhanced chondrogenesis, particularly as a cleavable peptide, when functionalised individually and in combination with N-cadherin or TGF-ß mimic peptides	(83, 87, 91, 103)
HAVDI	HA, KLD-12 peptide, PNIPAM -co-GMA	N-cadherin	Highly specific N-cadherin agonist derived from homophilic binding site ECD1	Human MSCs, rabbit MSCs	Enhanced chondrogenesis individually and in combination with RGD or GAG chondroitin sulfate, more effective when permanently tethered, regulates chondrogenesis through Wnt/ß-catenin signalling	(38, 80, 103, 109, 110, 120)
LRP5 peptide	Alginate	LRP5	Cell-cell interaction motif that binds N-cadherin	Mouse BMSCs	Facilitates stem cell aggregation to promote chondrogenesis at low seeding densities	(108)
SPPEPS	HA	TGF-ß3, Aggrecan	Acts as a ligand for various integrins that mediate cell-matrix signalling	Rat BMSCs	Enhances chondrogenesis individually and to a greater extent when combined with RGD	(87)
Cytomodulin-2	HA	TGF-ß	Interacts with cell surface TGF-β receptors	Human PLSCs	Promotes chondrogenesis	(88)
MMP7-cleavable peptide	Scl2		Recognised by MMP7 for degradability	Human MSCs	Positive correlation between degradability and cartilage matrix deposition	(16)
ADAMTS4-cleavable peptide	Scl2		Recognised by aggrecanases for degradability	Human MSCs	Positive correlation between degradability and cartilage matrix deposition	(16)
PFS bone marrow homing peptide	Acellular cartilage matrix		Identified by a phage display peptide library as a specific peptide that homes to bone marrow and binds to stem cells	Rabbit BMSCs	Enhanced rabbit BMSC homing and chondrogenesis	(79)

Table 2. Peptides used in recent studies for the functionalisation of hydrogels that regulate stem cell chondrogenesis.

2.4. Sustained release and retention of growth factors

Various growth factors are known to stimulate stem cell chondrogenic differentiation; these biomolecules have great potential for being administered clinically to promote cartilage tissue regeneration (121). However, without continuous local release of chondroinductive molecules at sites of interest, their effectiveness is significantly reduced. While some researcher have opted for the functionalisation of the hydrogels with growth factor mimicking peptides (as seen in section 2.3), others have developed growth factor-loaded hydrogels as potential tools to improve the local release and effectiveness of growth factors in cartilage tissue engineering. Indeed, recent studies have investigated ways of modifying hydrogel properties to improve the retention and sustained release of chondroinductive growth factors, namely TGF-ß, using either cell-laden or acellular systems.

2.4.1. Cell-laden hydrogels

A variety of approaches have been explored to improve the growth factor binding properties of hydrogels to sustain the differentiation of stem cells encapsulated within the matrix microenvironment, and, in particular their chondrogenesis (122). For example, Shen and co-authors showed that incorporation of GO nanosheets into PDLLA-PEG composite gels improved the sustained release of TGF-ß3 and maintained human BMSC chondrogenesis in an *in vivo* mouse model (123). PEG-poly(L-alanine)-poly(L-aspartate) triblock copolymer thermogels were also found to prolong the release of chondroinductive molecule kartogenin (KGN) when modified with RGD-functionalised hexagonal layered double hydroxides, improving human tonsil-derived MSC chondrogenesis (82).

TGF-ß family proteins are also known binding partners of HA, which makes HA-based gels a useful tool for studies into TGF-ß release and stem cell chondrogenesis. Hence, HA has gained interest not only as a GAG with known chondroinductive properties, but also as a reservoir for binding a variety of growth factors (124). Indeed, it has been shown that the inclusion of HA into PDLLA-PEG gel composites improves the sustained release of TGF-ß3 and maintenance of BMSC chondrogenesis *in vivo* (125). Sulfated HA was also shown to improve retention of TGF-ß1 and human MSC chondrogenesis *in vivo* in HA gels, compared to non-sulfated HA gels (126). These studies suggest that the inclusion and modification of HA in hydrogels has the potential to improve sustained GF release and chondrogenesis of stem cells, which can be beneficial in cartilage repair applications.

Physically assembled hydrogels are also of significant interest for the presentation of growth factors. This type of hydrogels more closely mimics the interconnectivity properties of native ECM, and possesses reversible crosslinking properties that are desirable in biomedical engineering, such as self-healing and shear-thinning (127-129). For example, host-guest macromer (HGM)-based crosslinking can generate physically assembled supramolecular hydrogels, whose GF release properties have been investigated for the maintenance of stem cell chondrogenesis. In particular, human MSC-laden HGM-based gels formed by molecular self-assembly between adamantane-functionalised HA guest polymers and monoacrylated ß-cyclodextrin host monomers were found to promote sustained TGF-ß release and chondrogenesis

in vivo compared to methacrylated HA gels (130). Similarly, gelatin-acrylated ß-cyclodextrin HGM-based gels improved the sustained release of KGN and TGF-ß, compared to GelMA, in turn enhancing hMSC chondrogenesis in an osteochondral defect mouse model *in vivo* (**Figure 4**) (131).

Besides the gel type, the mechanism of GF attachment to the hydrogels has also been investigated, in relation to how a transient or permanent interaction influences stem cell chondrogenesis. Indeed, HA/poly(glycidol) gels with covalently attached TGF-ß1 resulted in better human MSC chondrogenesis than non-covalent attachment (132).

Overall, these studies highlight the effect that the incorporation of growth factors within hydrogels has on the promotion of stem cell chondrogenesis, and suggest that a permanent tethering of chondroinductive growth factors, such as TGF-ß, may be more beneficial to sustain their effects on chondrogenic differentiation.

2.4.2. Acellular hydrogels

The development of acellular gels is advantageous over cell-laden gels as it removes the requirement for cell acquisition and encapsulation into hydrogels for *in vivo* applications. The properties of hydrogels can be modified to enhance the chondrogenic response of stem cells that are either already in the niche of interest or are recruited from other sites.

Using GF-loaded alginate/gelatin gels, it was shown that synergistic TGF-ß3/KGN delivery promoted MSC migration to the gels and enhanced chondrogenesis *in vivo* (133). Additionally, injectable thermosensitive chitosan gels were found to sustain the release of KGN over 40 days, enhancing human adipose-derived stem cell (ASC) chondrogenesis *in vitro* compared to soluble KGN (134). Alginate nanogels with a size range of 43-137 nm were also developed for sustained TGF-ß release, revealing that gels of the smallest size were most effective at GF release and maintenance of human MSC chondrogenesis *in vitro* (135). Glycidyl methacrylate hydroxypropyl chitin gels were shown to be effective at sustained TGF-ß1 release; this, activated macrophages to an M2 phenotype and improved rat MSC homing to an injury site and chondrogenesis *in vivo* (136).

Overall, these studies highlight the potential of acellular gels, either as bulk or nanogels, to improve sustained growth factor release and stem cell homing to support chondrogenesis at cartilage defect sites.

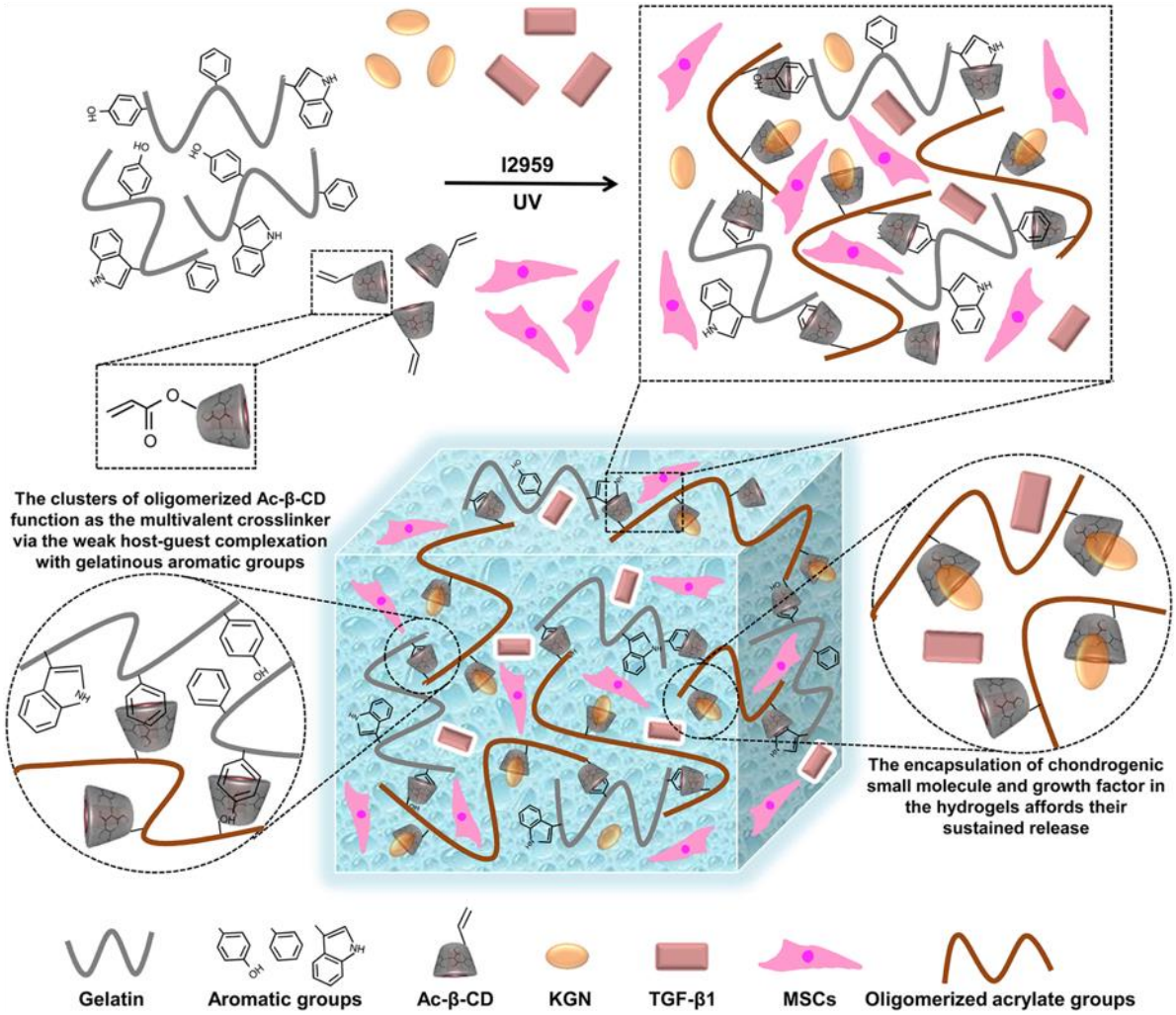

Figure 4. Example of cell-laden hydrogels developed to sustain growth factor release and promote maintenance of chondrogenesis. Schematic of MSC encapsulation in supramolecular gelatin hydrogels containing chondrogenic small molecules and growth factors. Reproduced with permission (131).

3. Mechanical properties

Cells are known to respond to a variety of different mechanical stimuli within their microenvironment; this influences their behaviour through mechanosensitive signalling. Hydrogel mechanical properties can be modified through a variety of techniques, such as adjusting polymer molecular weight and crosslinking; hence they can be tuned to maximise the mechanosensitive chondrogenic behaviour of stem cells. Additionally, the mechanical stability of hydrogel scaffolds is highly important in cartilage tissue engineering to withstand mechanical stresses when administered clinically and to effectively integrate with natural tissue.

3.1. Stiffness

Stiffness is the extent by which an object resists deformation in response to an applied force and is highly variable across different biological tissues (137). In the context of

stem cell mechanosensing, various studies have shown that hydrogel stiffness has a marked influence on human MSC differentiation. Perhaps the earliest, most comprehensive study used 2D PA hydrogels to show that, while soft matrices (0.1-1 kPa) mimicking brain tissue promoted neurogenesis, relatively stiffer gels (8-17 kPa) modelling muscle mechanical properties induced myogenesis, and the most rigid surfaces (25-40 kPa), more representative of collagenous bone, promoted osteogenesis (7).

Various recent works have investigated how modulating hydrogel stiffness influences stem cell chondrogenesis. Indeed, PLSCs expressed the chondrogenic marker collagen II in a stiffness-dependent manner: using alginate-HA gels at either 9 or 36 kPa resulted in lower expression than at a comparatively intermediate stiffness of 18 kPa (45). Another study showed that poly-L glutamic acid-tyramine hybrid gels at a stiffness of 4.2 kPa were more effective than stiffer 15.3 kPa gels at enhancing BMSC chondrogenesis *in vitro* and in an *in vivo* mouse model (81). Fibrin gels, prepared at various stiffnesses, were shown to be most optimal for ASC chondrogenesis at 3.4 kPa (61). Additionally, a study investigating different types of crosslinking for HA gels, showed that the stiffest formulation at 1.8 kPa, using 4-armed PEG crosslinking, was most optimal for BMSC chondrogenesis (37). Another study exploring hydrogel crosslinking options, showed that 9 kPa photo-crosslinked collagen I gels coincided with enhanced rabbit BMSC chondrogenesis both *in vitro* and *in vivo* in a mouse model, compared to softer, physically crosslinked 2 kPa gels (138). This study also highlighted the importance of actin-mediated matrix interactions during chondrogenesis by showing that inhibition of actin polymerisation with cytochalasin D worsened differentiation (138). Additionally, using PEG-GelMA hybrid gels, it was shown that a higher stiffness of 25 kPa was more effective than softer 1.6/6 kPa gels at promoting chondrogenesis of rabbit-isolated MSCs (102). Another study, using methacrylated HA gels, showed that a higher degree of methacrylation increased the hydrogel stiffness from 2 to 7 kPa, and this was more favourable for human ASC chondrogenesis (139).

Stiffness-specific differences between chondrogenic and osteogenic differentiation have also been shown recently, which are important considerations when designing biomaterials for osteochondral interface applications. This has been investigated using hydrogels with tuneable stiffness gradients, where gelatin-PNIPAM hydrogels, containing both beta-sheet rich and amorphous silk nanofiber solutions, were prepared by combining crosslinking and electric field alignment (77). This study showed that BMSC chondrogenesis, both *in vitro* and *in vivo* in a rat model, was enhanced in 23-64 kPa regions, before reducing at higher stiffnesses where osteogenesis was favoured (**Figure 5A**) (77).

3.1.1. Synergy with other cues

In general, hydrogels with an intermediate stiffness between soft and rigid gels, that promote neurogenesis or osteogenesis respectively (7), appear to be most suitable for supporting stem cell chondrogenesis. However, direct comparisons between studies are complex, since differences reported in stem cell chondrogenic behaviour are likely attributable to combinatory effects of stiffness and other factors, such as

seeding conditions (section 5.2.1), hypoxia (section 6.2), or reactive oxygen species (section 6.3).

The chondroinductive properties of the hydrogel material itself may synergistically influence chondrogenesis along with the mechanical environment. This is particularly relevant when using natural polymers, such as GAG-based gels, many of which have known chondroinductive properties that could play a role, compared to synthetic polymer-based gels (section 2.1). Indeed, it has been shown that GAG-functionalised PEG hydrogels, at a controllable stiffness of either 7.5 or 36 kPa, promoted human MSC chondrogenesis in softer gels better with CS modification, than HS, while stiff gels were inhibitory regardless of GAG presence (50). Another study showed that bioprinted alginate-gelatin gel scaffolds functionalised with CS displayed a stiffness of 59.7 kPa and supported BMSC neocartilage formation better than non-functionalised gels at 48 kPa (47). Interestingly, this study also outlined that the addition of HA, a known chondroinductive GAG, caused hypertrophic cartilage formation, as well as significantly increased stiffness to 100.1 kPa, perhaps suggesting that a densely crosslinked matrix hinders effective macromolecular diffusivity and cartilage development (47). Moreover, PEG gels functionalised with different GAGs and formed at different stiffnesses (1-33 kPa) showed that 7-33 kPa gels were required to maintain ASC chondrogenesis and gel integrity *in vivo*, highlighting the balance required between mechanical and biochemical properties for effective material development (51).

3.1.2. High-strength gels

Other studies have developed hydrogel-based scaffolds for stem cell chondrogenesis at high stiffness ranges, closer to the values of native cartilage tissue; in this case, the objective is to manufacture gels with a mechanical strength that would be suitable for point-of-care cartilage defect treatments in clinical practice (140). The target stiffness range for developing high-strength gels are those that mimic the compressive moduli of native human articular cartilage tissue which ranges from 240 to 1000 kPa (141, 142) and is an order of magnitude higher than hydrogels typically produced from natural materials (143). One approach, using PDLLA-PEG hydrogels, increased the stiffness from 200 to 250 kPa by incorporating graphene oxide (GO) nanosheets, which enhanced human MSC chondrogenesis (67). PDLLA-PEG gels were further shown to achieve a stiffness of 300 kPa at lower polymer concentrations and enhance BMSC chondrogenesis compared to poly L-lactic acid (PLLA)-PEG gels (2). Other high stiffness gels that were found to be conducive to stem cell chondrogenesis include citric acid-crosslinked chitosan gels, with a stiffness of 500 kPa compared to the 50 kPa of unmodified chitosan (63). Stem cell chondrogenesis was also enhanced using alginate-PRP gel composites (148 kPa compared to 133 kPa of alginate only gels) (56) and methacrylated decellularized porcine cartilage gels (250 kPa compared to 50 kPa of GelMa gels of the same relative composition) (53).

Previous seminal studies have shown that stiff environments are more osteogenic compared to softer chondroinductive materials (7, 77); this would suggest that it is unlikely for stem cell chondrogenesis in high-strength gels to be stiffness-driven. However, recent research has highlighted biphasic relationships between cell

behaviour and substrate stiffness. Indeed, Yes-associated protein (YAP) mechanosensing shows a biphasic response depending on both substrate stiffness and RGD ligand spacing (144). Since YAP is a crucial mechanosensitive transcriptional coactivator involved in regulating cell behaviour, such as differentiation, this could be associated with how high-strength gels influence chondrogenesis. Other studies have also shown biphasic relationships between substrate stiffness, ligand density, and various types of cell behaviour, including migration (145) and proliferation (146). Whether a biphasic response to rigidity promotes stem cell chondrogenesis in high-strength gels is however unclear and would be interesting to explore. Ultimately, the chondrogenic response in these gels is likely to be driven by a combination of factors alongside the high mechanical strength. These cues may include the biochemical and microstructural properties of the material components used in the hydrogel fabrication. In any case, high-strength hydrogels or composites of softer chondrogenic gels with structural materials can recapitulate the mechanical properties of native cartilage tissue and are more likely to withstand mechanical stresses during long-term cartilage repair applications.

3.2. Viscoelasticity

When investigating stem cell differentiation due to mechanical cues, most hydrogel studies to date have used elastic materials with controllable stiffness or have disregarded their viscous component. Although these studies provide fundamental insights into our understanding of how the mechanical microenvironment can influence chondrogenesis, they do not fully represent the properties of native cell surroundings. Indeed, most biological tissues are viscoelastic, meaning they exhibit both elastic and viscous properties. Viscoelastic materials display a time-dependant deformation following the application of a force, which is recovered with a timescale that depends on the extent of viscoelasticity. Conversely, elastic substrates store mechanical energy and respond to an applied stress with time-independent strain, where the material immediately returns to its original structure upon removal of the stress. Generating hydrogels with controllable viscoelastic properties has great potential in harnessing the mechanosensitive response of stem cells, also in the field of chondrogenesis (147, 148).

This is a relatively new area of research, meaning much is yet to be understood about cell response to such environments. Steric spacing of crosslinking points and incorporation of viscous, linear polymers are examples of strategies to modulate hydrogel viscoelasticity, which has a significant influence on key cell behavioural aspects, such as adhesion and differentiation (149, 150). In the field of cartilage engineering, a study, using bovine chondrocytes, showed that alginate gels with variable viscoelasticity, at a controllable stiffness of ~3 kPa, promoted cartilage matrix deposition on gels with increasing viscoelasticity (**Figure 5B**) (151).

Stem cell chondrogenesis in viscoelastic environments is relatively unexplored; PEG-gellan gum hybrid gels with enhanced viscoelastic gel properties compared to an elastic PEG control were shown to promote BMSC chondrogenesis, both *in vitro* and *in vivo* in a mouse model (152). However, it is worth noting that in this study hydrogel stiffness, as well as viscoelasticity, increased relative to the elastic control; this makes

it difficult to conclude whether changes in chondrogenesis were a consequence of stiffness, viscoelasticity, or both. Similarly, in a study using mouse chondrocytes, the chondroinductive potential of viscoelastic hydrogels was shown using physically crosslinked composite hyaluronate-alginate hydrogels. Increasing the molecular weight of hyaluronate whilst maintaining a constant alginate molecular weight increased the elastic and viscous moduli of the hydrogels. This enhanced chondrogenesis with significantly higher expression of pro-chondrogenic markers Sox9 and collagen-II (153). While both of these studies indicate that viscous interactions play a role in chondrogenesis, the viscous contribution is accompanied by a concomitant increase in elastic modulus; this makes it impossible to establish whether the improved chondrogenesis is a consequence of the viscous component alone.

Besides viscoelastic hydrogels, recent research has also investigated cell response to viscosity in viscous fluids. In the case of chondrogenesis, Lee and co-authors recently developed a 3D culture system where viscous gelatin solutions could be incorporated into GelMA hydrogels. They found that hydrogels embedded with viscous gelatin improved spreading and proliferation of hMSCs compared to a stiff gelatin hydrogel. Furthermore, increasing gelatin solution viscosity significantly enhanced production of sulphated GAGs, a sign of chondrogenic differentiation. Further investigation into chondrogenic gene expression demonstrated that, in the presence of chondrogenic induction factors dexamethasone and TGF-β3, chondrogenic differentiation was enhanced with increasing gelatin solution viscosity. However, in the absence of chondroinductive factors, this trend was not seen, suggesting that viscosity alone was not sufficient to promote chondrogenesis (154). These results point to a synergistic effect between solution viscosity and biochemical induction on hMSC chondrogenesis.

Although research into the role of viscous interaction in chondrogenic differentiation is still limited, collectively these studies demonstrate the importance of incorporating viscoelastic properties into hydrogel design to better mimic ECM biomechanical properties and subsequently enhance stem cell chondrogenesis.

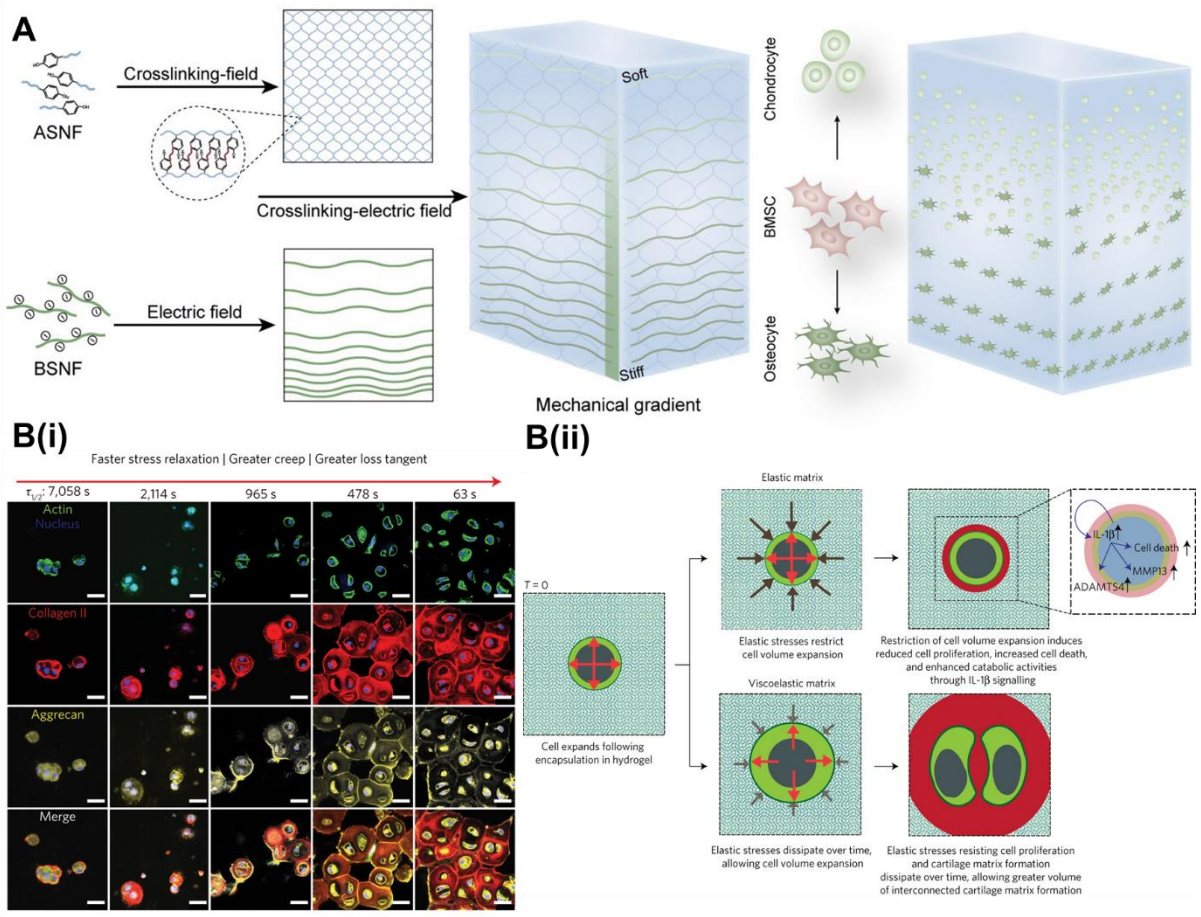

Figure 5. Elastic and viscous mechanical properties of hydrogels that regulate chondrogenesis. A) Preparation of silk nanofiber hydrogels with stiffness gradients that control the chondrogenic and osteogenic fate of BMSCs. Reproduced with permission (77). **B)** Immunohistochemical staining of cartilage matrix markers following chondrocyte culture in 3 kPa hydrogels with variable viscoelasticity **(i).** Model of how viscoelastic matrices dissipate elastic stresses to promote cell volume expansion, cell proliferation, and interconnected cartilage matrix deposition **(ii)**. Reproduced with permission (151).

4. Microstructural and spatial properties

The architecture of hydrogels has been shown in various studies to significantly influence the chondrogenic differentiation of stem cells. Since the naturally occurring cellular microenvironment is highly complex and diverse in its structure, it is important to understand the types of structural features that can modulate stem cell chondrogenesis with the objective of developing effective biomaterials for cartilage tissue engineering.

4.1. Porosity

Changes in the hydrogel porous structure are generally brought about through altering composition or types of polymer and crosslinking within hydrogels. It is worth noting that changes in porosity will likely correlate with changes in the mechanical properties

of the hydrogels as well, which are known to significantly influence chondrogenesis, and therefore it is likely they collectively modulate the cell response.

It has been shown that stem cells with a more rounded morphology are associated with higher expression of chondrogenic markers (155, 156). Hence, tuning material porosity and pore structure to direct cell shape is an interesting strategy to artificially induce stem cell chondrogenesis and promote matrix secretion and deposition. Indeed, collagen scaffolds with a more elliptical pore shape were found to promote BMSC chondrogenesis (157). Pore size is also important, and it has been suggested that pores between 50-300 μm are within a suitable size range for stimulating cartilage regeneration. Indeed, BMSC chondrogenesis was enhanced in collagen scaffolds containing the highest proportion of pores within this size range (157). It has also been shown using collagen-HA scaffolds that pores of 300 μm were optimal for MSC chondrogenesis compared with smaller 94 and 130 μm sizes (**Figure 6A**) (158). Additionally, using collagen matrices, a higher pore size of ~80 μm vs 20 μm improved chondrocyte maintenance (159). Using poly-L-glutamic acid/tyramine-based gels, 2% porous gels were shown to have a larger porous structure (92 ± 11 μm), which was more favourable for BMSC chondrogenesis, both *in vitro* and *in vivo* in a mouse model, compared to 4% gels that had a more compact network structure (54 ± 17 μm) (81).

Other features besides pore size and shape, including porosity, pore uniformity, and pore interconnectivity, have been highlighted as important in the regulation of stem cell chondrogenesis. Indeed, increasing the porosity of chitosan gels from 80.5% ± 0.9% to 89.2% ± 1.3% via citric acid crosslinking enhanced chondrogenesis of human MSCs; this was also correlated with increased pore interconnectivity and pore wall strength (63). Additionally, alginate gels with a pore size of 200-300 μm displayed higher pore uniformity and pore surface roughness when functionalised with PRP; this in turn enhanced BMSC chondrogenesis (56). These studies suggest that, as well as an ideal pore size, uniformity and nanotopographical features of the pores are important for regulating the chondrogenic fate of stem cells. Additionally, gelatin-based gels with a microribbon (μRB) structure, which confers them a cell-scale macroporosity, were shown to enhance human MSC chondrogenesis compared to conventional gels which lack such features. Specifically, MSC-seeded μRB scaffolds exhibited a 20-fold increase in compressive modulus to 225 kPa after 21 days, a range that is approaching the level of native cartilage, compared to control gels that only modestly increased to 65 kPa (160). Unlike conventional microporous hydrogels, the highly interconnected macroporous structure of μRB scaffolds display shock absorbing characteristics through a spring-like mechanical property upon compression (161). High μRB pore interconnectivity combined with its unique mechanical properties could then be an attractive system to facilitate stem cell chondrogenesis for articular cartilage repair.

Interestingly, other studies have shown that an increase in porous features might not be necessarily beneficial for chondrogenesis. Indeed, using HA gels formed using different crosslinking approaches, BMSC chondrogenesis was enhanced the most using 4-armed PEG crosslinking, which generated gels with the largest range of pore sizes from 100-200 μm, but also with comparatively lower porosity (37). A reduced pore size from 971 to 391 μm and flatter pore surface were found to promote

chondrogenesis of human ASCs using photopolymerisable methacrylated HA gels with greater degrees of methacrylation (139). Photo-crosslinked collagen I gels with reduced porosity compared to their physically crosslinked counterparts (from 52% to 40%) , also showed an increase in rabbit BMSC chondrogenesis, both *in vitro* and *in vivo* (138).

Clearly, there is not a linear relationship between hydrogel porous features and stem cell chondrogenesis, rather there is an optimal range in which these features must be tuned to optimise the cell response. Generally, high pore uniformity and interconnectivity appear to promote chondrogenesis, as well as an optimal pore size range and porosity.

4.2. Topographical features and ligand patterning

It has been shown in various studies that the presentation of micro and nanotopographical features can significantly influence MSC mechanosensing and regulate their spreading and differentiation behaviour (162). However, most of these works use rigid materials, such as TiO_2, and investigate osteogenesis (163, 164). Fewer studies have tackled the addition of topographical features on hydrogels, likely due to the difficulty to fabricate fine nanoscale features on swelling materials with low mechanical moduli (165).

Cao et al. managed to pattern 30 µm and 60 µm microislands onto PEG gels, demonstrating a greater chondrogenic response of rat BMSCs in the smaller islands, where cell spreading is limited (**Figure 6B**) (166). Thermosensitive chitosan/PNIPAM gels with 50 µm microstripes were also shown to promote a more organised chondrogenic response from mouse MSCs, which mimicked that of the superficial zone of cartilage tissue, compared to unpatterned substrates which more closely resembled the middle cartilage zone (76). This suggests that surface patterning could be used in anisotropic materials to develop zonal cartilage tissues, where topography in each zone can be tuned to direct the chondrogenic response. Similarly, Kim and co-authors introduced spatial features within hydrogels by developing fibrous HA hydrogels, suggesting that their tuneable mechanics and adhesivity make them a promising alternative to non-fibrous hydrogels (167). Yang et al. also reported that collagen gels with a fibrous architecture, compared to porous gels, enhanced BMSC chondrogenesis both *in vitro* and *in vivo* (17).

While the features discussed above are at the microscale, nanotopograhical cues have also been suggested to regulate chondrogenesis. For example, roughness of the pore surface of a gel has been implicated in directing the chondrogenic fate: alginate hydrogels with incorporated PRP displayed increased roughness, which could be a contributing factor in the enhanced chondrogenic response observed from mice BMSCs (56). Additionally, nanorods functionalised to HA gels were shown to enhance BMSC chondrogenesis by generating a highly ordered nanotopographical environment (168).

Besides using topographical cues, the patterning of adhesive ligands, such as RGD, has shown potential in directing stem cell chondrogenesis. For example, hexagonal patterns of RGD on PEG gels elicited differences in MSC behaviour based on ligand

spacing, where chondrogenesis was favoured on gels presenting larger 161 nm RGD spacing compared to a 63 nm spacing (169). Interestingly, the larger nanospacing prompted reduced MSC spreading; this is in agreement with previous work that has shown that a small spreading area, for example via constraining cells in microislands (166), is beneficial for stem cell chondrogenesis.

4.3. Zonal hydrogels

Native articular cartilage has a zonal organisation, with depth-dependent ECM properties. These physiological spatial features have inspired the design of anisotropic hydrogels, where a gradient of microstructural and mechanical properties results in a local control of stem cell differentiation. This approach has instructed the development of hydrogels for osteochondral regeneration, and for the regeneration of zonally organised cartilage.

For example, Xu et al. developed an osteochondral material based on gelatin-PNIPAM hydrogels containing both beta-sheet rich and amorphous silk solutions that displayed a tuneable stiffness gradient. By combining crosslinking and electric field alignment, the authors recapitulated softer and stiffer region-specific properties that respectively promoted chondrogenesis or osteogenesis of human MSCs *in vitro* and stimulated ectopic osteochondral tissue regeneration *in vivo* (**Figure 5A**) (77).

Gegg and Yang showed that anisotropic gels could be used to reproduce the different zones of native cartilage tissue using spatially patterned µRB-based gelatin-CS gels. Mechanical and biochemical properties were tuned by varying gelatin/CS ratios, where human MSCs in the superficial zone showed less GAGs and more collagen relative to middle/deep zones. µRB samples significantly increased stiffness towards native tissue properties compared to isotropic gels, suggesting µRB approaches could be advantageous for anisotropic cartilage tissue interfaces (170). Recapitulating the different regions of native cartilage tissue has also been shown using a three-layer PEG-based hydrogel, functionalised with CS, HA and MMP-sensitive regions in specific combinations to represent different zones. The superficial zone was composed of PEG, CS, and MMP-sensitive regions, the middle zone contained PEG and CS, and the deep zone with PEG and HA. This directed BMSC chondrogenesis to create native-like articular cartilage with spatially distinct mechanical and biochemical properties (**Figure 6C**) (171).

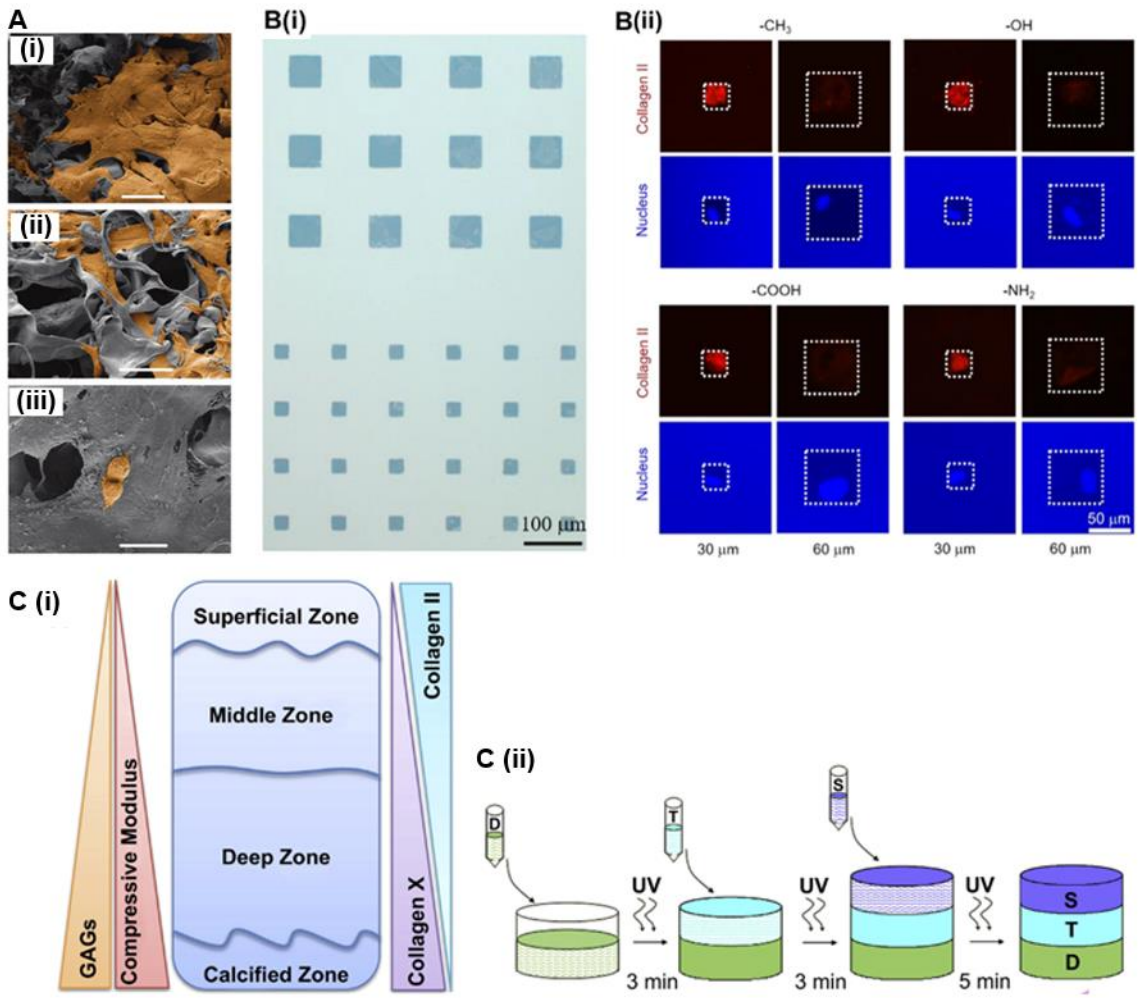

Figure 6. Microstructural and spatial hydrogel cues that regulate stem cell chondrogenesis. A) Scanning electron micrographs of a collagen-HA scaffold with varying mean pore sizes: 94 µm **(i)**, 130 µm **(ii)**, and 300 µm **(iii)** seeded with rat derived MSCs for 24 h. Cells appear flatly attached on the 94 and 130 µm mean pore size scaffolds, whereas cells on 300 µm mean pore size scaffolds attached with a rounded morphology which favoured chondrogenesis. Scale bar represents 20 µm. Reproduced with permission (158). **B)** Bright-field micrograph of a micropatterned PEG gel with squared microislands of side lengths 30 and 60 µm **(i).** Micrographs of collagen II staining from individual MSCs after 9 days of chondrogenic induction on the micropatterned PEG surfaces with different functional group modifications **(ii).** Reproduced with permission (166). **C)** Schematic of articular cartilage anatomy illustrating the mechanical anisotropy and distribution of GAGs, collagen II, and collagen X **(i).** Multi-layered hydrogel fabrication with three distinctive layers, each corresponding to the superficial, transitional, and deep zones of articular cartilage **(ii).** Reproduced with permission (171).

5. Cellular content

Besides the significant influence of hydrogel properties on stem cell behaviour, the cellular content within the hydrogel plays a crucial role in driving the chondrogenic response. Cell-cell mediated behaviour can be through physical interactions between cells, as well as via biochemical signalling following secretion of soluble factors. Modulating the cellular content of hydrogel systems has great potential in harnessing stem cell chondrogenic responses through the combinatory effects of cell-matrix interactions and cell-cell communication.

5.1. Seeding density

Optimisation of stem cell seeding densities has been shown to be a key factor for controlling chondrogenesis, as specific spatial distribution and organisation of cells in hydrogels can be conducive to a more chondrogenic phenotype. Indeed, a seeding density of 20 x 10^6 cells/mL was found to be optimal for BMSC chondrogenic matrix secretion within PDLLA-PEG gels (2). Using TGF-ß3-functionalised PEG gels, another study tested a range of BMSC seeding densities between 1.6 x 10^6 and 50 x 10^6 cells/mL and found that densities below 12 x 10^6 cells/mL were suboptimal for chondrogenesis while that higher densities enhanced chondrogenesis and endochondral ossification in mice (172). Additionally, 5 x 10^6 cells/mL was found to be an optimal seeding density for human BMSC chondrogenesis in collagen/alginate gels (35).

Ha et al. used an *in vivo* rabbit model to study how human umbilical cord-derived MSC seeding density in HA gels influenced cartilage repair over time. While a density of 5 x 10^6 cells/mL showed the highest cartilage repair at 4- and 16-weeks post-implantation, compared to the lower seeding density of 1 x 10^6 cells/mL, the lower density gave the highest score at 8 weeks post-implantation. This reflects how specific seeding densities in hydrogels are appropriate for chondrogenesis at certain time points during cartilage regeneration. Moreover, a higher density of 15 x 10^6 cells/mL was found to be not favourable for cartilage repair (173).

While the seeding densities used in these studies are all within the order of magnitude of 10^6-10^7 cells/mL, optimal values vary, showing that it is impractical to determine an optimal seeding density for all hydrogel systems, due to the influence of the various hydrogel materials and their properties.

5.2. Cell-cell contacts

Cell-cell contacts are thought to be highly significant events in mesenchymal condensation and chondrogenic differentiation. Indeed, the gold standard for MSC chondrogenesis uses pellet culture, and various studies have highlighted instances where cell-cell contacts between stem cells have coincided with an enhanced chondrogenic response. Using HA gels, enhanced human MSC chondrogenesis and nuclear ß-catenin localisation was observed in the case of clustering compared to individual cells (**Figure 7A**) (110). Similarly, PEG-poly(L-alanine)-poly(L- aspartate) triblock copolymer thermogels modified with RGD-functionalised hexagonal layered

double hydroxides were found to enhance human tonsil-derived MSC chondrogenesis through greater cell-cell contacts and aggregate formation (82).

As anticipated in section 2.3.2, using hydrogels functionalised with peptides that encourage cell-cell communication, such as N-cadherin motifs, is a promising approach to minimise the numbers of cells required for cartilage regeneration. Indeed, chondrogenesis of mouse BMSCs was enhanced in alginate gels functionalised with N-cadherin motif LRP5; this functionalisation was particularly critical for chondrogenic maintenance when cell density was low (108).

5.2.1. Microspheres

Incorporating stem cells into hydrogels as microspheres, rather than single cells suspensions, is another approach that could present a more chondrogenic environment by encouraging cell-cell communication. Indeed, chondrogenesis was enhanced when 10 x 10^6 million periosteum-derived stem cells/mL were seeded in collagen gels as microaggregates compared to single cells (174). Similarly, rabbit MSC chondrogenesis was increased when they were encapsulated as microspheres in collagen gels, compared to bulk, single cell encapsulation, or non-encapsulated cell pellets (175). Žigon-Branc et al. also found that soft 538 Pa GelMA gels enhanced human ASC microspheroid chondrogenesis, compared to stiffer 3584 or 7263 Pa gels (25).

Contrary to these studies, Rogan and co-authors found that single cell encapsulation of human BMSCs was more beneficial than micropellets for chondrogenesis in a variety of different GAG-functionalised hydrogels (176). This further highlights how stem cell chondrogenesis is largely driven by a synergy between cellular conditions and hydrogel-specific properties. It is however evident that, in some systems, stem cell microspheres are advantageous for chondrogenesis, likely because they provide a 3D microenvironment that better enables cell-cell communication.

5.3. Co-cultures

Hydrogels have also been used as microenvironments for the co-culture of stem cells with chondrocytes. The inclusion of chondrocytes, which are an integral component of cartilage, is thought to promote stem cell chondrogenesis through cell-cell signalling. Indeed, the addition of ACs to human MSCs in polyvinyl alcohol (PVA)-based hydrogels under hypoxic conditions enhanced the MSC chondrogenic response (177). Similarly, the presence of human ACs in co-culture with ASCs in PEG gels catalysed the production of neocartilage, suggesting that stem cell-chondrocyte co-cultures could help minimise fibrocartilage formation (178). Optimising the ratio between stem cells and chondrocytes is also important to optimise cartilage regeneration: Amann et al. found that a 1:3 ASCs:ACs ratio gave the best chondrogenic response in HA-collagen gels (**Figure 7B**) (179).

Overall, these studies suggest that co-cultures of stem cells with chondrocytes can be beneficial in harnessing the chondrogenic response within hydrogels. However, this should controlled and optimised, not only in terms of cellular ratios, but also in combination with suitable mechanical and biochemical hydrogel properties (51).

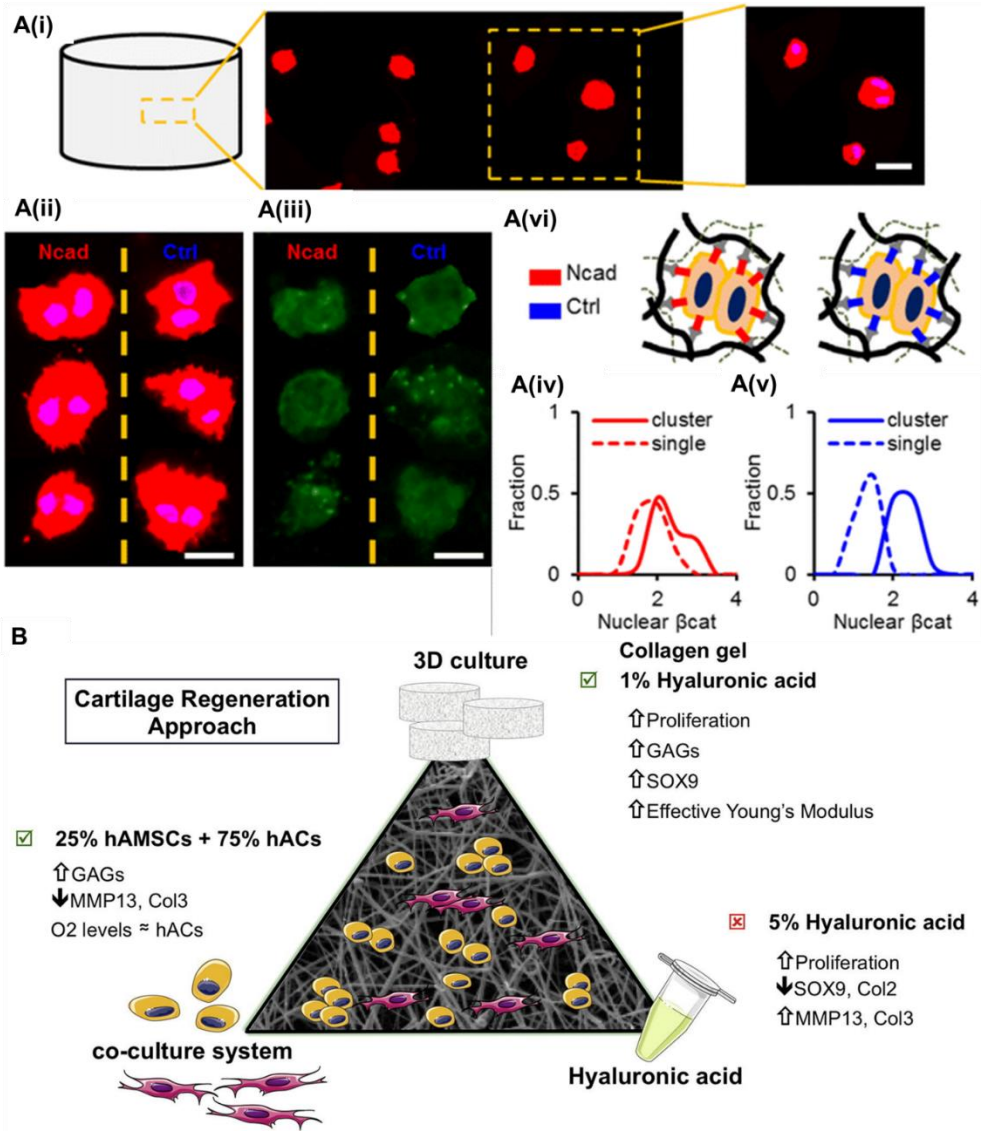

Figure 7. Influence of the cellular microenvironment on chondrogenesis in hydrogels. A) Representative single- and two-cell clusters within a hydrogel **(i).** Representative images of two-cell clusters in N-cadherin (left) and control (right) hydrogels stained for actin (red) and nuclei (blue) **(ii).** Average projections of the same clusters immunostained for β-catenin (green) **(iii).** Histograms of nuclear β-catenin frequencies in two-cell clusters (solid lines) vs. single MSCs (dashed lines) in N-cadherin hydrogels (red) **(iv)** and control hydrogels (blue) **(v)**. **(vi)** Schematic of cell-cell and cell-hydrogel interactions in hydrogels containing either Ncad or Ctrl peptides. Scale bars are 25 μm. Reproduced with permission (110). **B)** Graphical representation of hydrogel with optimised cellular and material composition for chondrogenesis using collagen-HA composite gels containing 1% HA and a ratio of 25% MSCs with 75% chondrocytes. Reproduced with permission (179).

6. External factors

Alongside hydrogel properties and cellular content, various external factors can act upon stem cells to direct their chondrogenic fate.

6.1. Mechanical loading

Mechanical stimulation of stem cell-laden hydrogels by physically applying an external stress has been shown in various studies to influence stem cell chondrogenesis. Indeed, mechanical compression was found to increase the chondrogenesis of human MSCs in porous polyurethane-methylcellulose composite hydrogel scaffolds (180). Similarly, dynamic mechanical loading was shown to enhance chondrogenesis of rabbit BMSCs in collagen gels (181), and to precondition rat MSC-laden HA gels for enhanced chondrogenesis *in vivo* (182). As with other chondrogenic cues, mechanical loading conditions should be optimised to ensure that the desired chondrogenic response is achieved; for example, Kowsari-Esfahan et al. investigated the effect of a range of mechanical strains on cell-laden alginate gels and found that 10% strain was the optimal condition for chondrogenesis of rabbit ASCs (183).

Recent research has linked mechanical loading to the inhibition of unwanted hypertrophy during chondrogenesis. Indeed, Aisenbrey and Bryant showed that dynamic loading under different moderate strains prevented the occurrence of an hypertrophic phenotype in MSCs cultured within chondrogenic RGD- and CS-functionalised PEG gels, compared to cells in unloaded gels (184). The authors uncovered that mechanical loading modulates mechanosensitive signalling to direct chondrogenic fate through hypertrophic inhibition. Using CS-containing PEG-norbornene-based gels, it was shown that human iPSC chondrogenic hypertrophy was regulated through CS-mediated inhibition of Smad1/5/8 signalling and upregulation of p38 signalling under dynamic loading (**Figure 8A**) (185, 186), as further elucidated in section 7.1. Mechanical stimulation was also applied to MSCs within HA gels using low intensity ultrasounds, showing an increase in collagen II and CS deposition compared to non-stimulated cell-laden gels (187). This effect was related to an increase in extracellular signal-regulated kinases 1/2 (ERK1/2) signalling and a disruption of the actin cytoskeleton.

Taken together, these studies indicate that mechanical stimulation can be a great tool to control stem cell chondrogenesis in stem cell-laden gels. Importantly, they confirm that the regulation of crucial mechanosensitive signalling pathways is key to the attainment of the stable non-hypertrophic chondrogenic phenotype that is needed in cartilage tissue engineering.

6.2. Hypoxia

Hypoxia is known to play a key role throughout life in regulating chondrogenesis and chondrocyte maintenance through hypoxia inducible factor (HIF)-mediated cellular responses (188, 189). It is thought that hypoxic conditions play a synergistic role, as an external environmental factor, with other microenvironmental cues to regulate the stem cell chondrogenic response. This has been modelled using hydrogels in a variety of studies. For example, human MSCs on soft ~1 kPa PAAM gels in hypoxic conditions

showed enhanced Rho-associated kinase (ROCK)-mediated chondrogenesis compared to normoxic or stiffer conditions (**Figure 8B**) (69). This highlights the importance of the synergy between mechanosensitive and hypoxic cell responses in regulating chondrogenesis. Further cues have been revealed to act in combination with hypoxia to enhance chondrogenesis; for example, Huang et al. showed that, while human MSCs in PVA-based gels underwent enhanced chondrogenesis in hypoxic conditions, this was further increased when MSCs were co-cultured with chondrocytes, indicating combinatory hypoxic and cell-cell effects (177). This was confirmed by Amann et al., who found that oxygen levels were lowest in chondrogenically-optimised co-cultures of human ASCs and ACs in HA/collagen-based gels (179). Sathy and co-authors revealed that hypoxic conditions could be mimicked using HIF-1α inhibitor dimethyloxalylglycine (DMOG): dose-dependent DMOG loading into pig MSC-laden alginate gels increased chondrogenesis and inhibited hypertrophy through increased Smad2/3 nuclear localisation. This was further enhanced *in vivo* through co-delivery with bone morphogenic protein-2 (BMP-2) and TGF-ß3 (190), suggesting that the combination of biochemical manipulation and hypoxic conditions can be effective in directing MSC chondrogenic fate. Finally, hypoxic environments were found to inhibit chondrogenic hypertrophy and calcification of ASCs during bone-tendon interface integration using bioprinted hydroxyapatite-functionalised HA gels (191).

Controlled hypoxic conditions are therefore an important parameter that can facilitate stem cell chondrogenesis and osteochondral integration in combination with mechanosensitive and biochemical processes in hydrogel-based microenvironments.

6.3. Stimuli-responsive particles

The use of small, stimuli-responsive particles has been the subject of various fields of research into cellular responses mediated by triggered particle activation. Specifically, upon the application of certain environmental stimuli, particle components can interact to bring about different phenomena, such as chemical exchanges, energy transfer, mechanical work, and changes in physical properties (192). Some of these particle systems have been incorporated into hydrogel-based materials and investigated in the context of stem cell chondrogenesis.

For example, photosensitive particles can respond to light-based stimuli, and in turn cause changes in the local environment. Certain types of particles, in response to light, can generate reactive oxygen species (ROS), which have been shown to influence BMSC differentiation at appropriate levels (193). Using BMSC-laden collagen gels conjugated with photosensitive particles, it was shown that photodynamic therapy (PDT)-induced ROS formation enhanced chondrogenesis through changes in mammalian target of rapamycin (mTOR)-mediated signalling, both *in vitro* and *in vivo* in a cartilage defect mouse model (18, 194). Additionally, particle conjugation increased hydrogel stiffness, and this in turn was found to influence chondrogenesis through TGF-ß/Smad-mediated signalling, as further elucidated in section 7.1 (18, 194).

The use of magnetically-responsive nanoparticles has also been explored in few studies addressing how magnetic manipulation of nanoparticles encapsulated in

hydrogels can influence stem cell chondrogenesis. Mechanical agitation of these magnetically-responsive nanoparticles can be achieved through the application of an external electromagnetic field. For example, Popa and co-authors showed that magnetic nanoparticle-loaded k-carregeenan-based polysaccharide gels could stimulate human ASC chondrogenesis upon the application of a magnetic field (195). Additionally, gelatin hydrogels loaded with magnetic particles were shown to promote rat BMSC chondrogenesis under magnetic field stimulation (196).

Taken together, these studies show that the incorporation of stimuli-responsive particles within hydrogels for stem cell-based cartilage engineering has an interesting potential for the development of systems that can be controlled with an external trigger. While PDT-induced ROS formation showed that ROS could act in synergy with mechanosensitive pathways to regulate chondrogenesis in hydrogel microenvironments, the underlying mechanisms by which magnetic stimulation elicits a chondrogenic response from stem cells remain relatively unexplored.

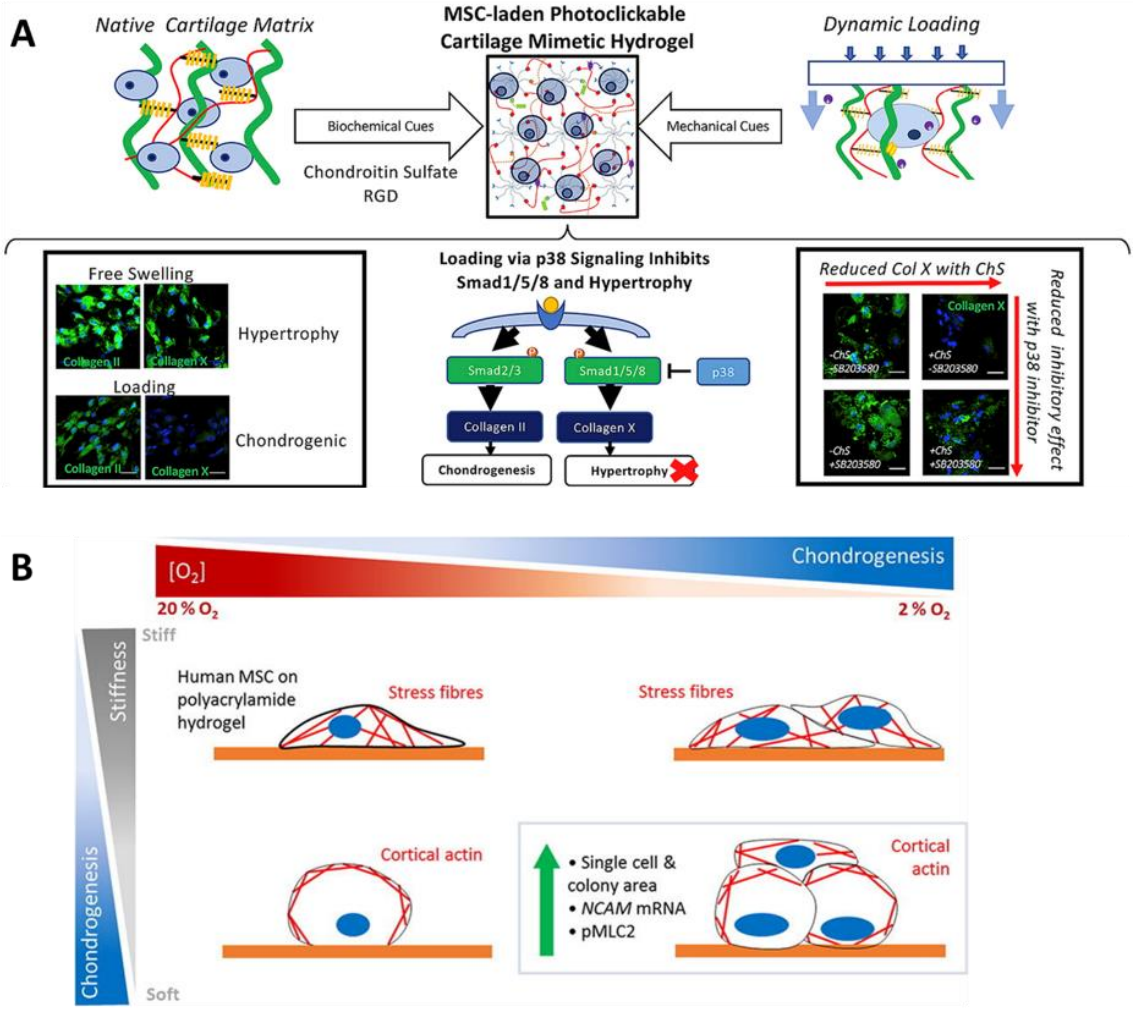

Figure 8. External mechanical and biochemical cues that regulate stem cell chondrogenesis in hydrogels. A) Synergistic biochemical and mechanical cues of CS-modified gels under dynamic loading regulate stem cell chondrogenesis by suppressing hypertrophy through Smad1/5/8 inhibition. Reproduced with permission (186). **B)** Hypoxia influences chondrogenesis in a mechanosensitive manner by regulating cytoskeletal tension. Reproduced with permission (69).

7. Signalling pathways

Several signalling pathways converge to regulate stem cell chondrogenesis, and their manipulation can help direct cell fate. Some of the main pathways involved include fibroblast growth factor (FGF), TGF-ß/BMP/Smad, Wnt/ß-catenin, Hedgehog, and Notch signalling, as well as hypoxic and angiogenic-related pathways. For an in-depth analysis of these signalling regulators in chondrogenesis, the reader can refer to recent reviews, such as (197). The transcription factor Sox9 is arguably the most important regulator of chondrogenesis; its high expression is crucial for the maintenance of chondrocyte phenotype (198). Proteins involved in chondrogenic development, such as collagen II and aggrecan, are regulated by Sox9 through its direct binding and activation of their promotor elements (199-201). Moreover, Sox9 has been shown to converge with many of the pathways involved in regulating chondrogenesis and is therefore a key downstream regulator. Indeed, FGF2 induces chondrocyte proliferation through upregulation of Sox9 (202, 203), which then potentiates the prochondrogenic properties of BMP2 whilst inhibiting BMP2-induced osteogenesis and endochondral ossification (204). Additionally, various co-activators of Sox9 expression, such as Smad 2 and 3 (205), have been identified in MSCs; this further highlights the regulatory role of Sox9 during chondrogenesis downstream of certain pathways. Sox9 is also involved in Wnt/ß-catenin signalling by targeting promotors for degradation by ubiquitination/proteasomes (206, 207). The main antagonist of Sox9 is runx-related transcription factor 2 (Runx2), a transcription factor that is the primary regulator of osteogenesis. High Runx2 expression blunts Sox9 activity (207), while increased Sox9 levels depress Runx2 expression amongst other pro-osteogenic proteins (204, 208, 209). Another negative regulator of Sox9 is RhoA (210), which is heavily involved in regulating cytoskeletal dynamics through specific downstream effectors that control actin organisation, namely ROCK. This highlights how Sox9 activity is likely sensitive to changes in the mechanical environment that influence cytoskeletal properties following cell-matrix interactions.

While many studies have used conventional 2D surfaces or pellet culture systems to investigate chondrogenic signalling, both of these systems fail to present cells with an appropriate microenvironment. Here, we will review the main stem cell chondrogenesis pathways that have been targeted via hydrogel cues, which, as we have elucidated in the previous sections, can more closely mimic the native ECM.

7.1. TGF-ß/Smad signalling

TGF-ß signalling is involved in a variety of cell processes, such as growth, apoptosis, and differentiation. The pathway functions through regulation of Smad proteins, where TGF-ß family ligand-receptor binding initiates a cascade to facilitate phosphorylation and subsequent binding/activation of Smad proteins. This regulates gene expression, for example through Sox9, a key downstream effector of Smad signalling (204, 205). Using hydrogels as ECM mimics, various studies have highlighted the role of TGF-ß/Smad signalling in stem cell chondrogenesis.

As anticipated in section 6.1, Aisenbrey and Bryant showed that increased chondrogenesis of iPSCs in CS-containing PEG-norbornene gels under dynamic

mechanical loading was regulated by CS-mediated inhibition of Smad1/5/8 and upregulation of p38 signalling (186). p38 family molecules are stress responsive constituents of the mitogen-activated protein kinase (MAPK) signalling pathway: this suggests a mechanosensitive crosstalk between p38 MAPKs and TGF-ß/Smad signalling in stem cell chondrogenesis. Indeed, previous work has shown that inhibition of p38 MAPK signalling attenuated expression of TGF-ß receptors and Smad3 (211). Further work by the same research group showed that the synergy of mechanical (dynamic loading) and biochemical (exogenous TGF-ß3) effects caused Smad-mediated inhibition of chondrogenic hypertrophy through enhanced signalling of Smad 2/3 over Smad1/5/8 in PEG-norbornene gels, suggesting preferential activation of specific Smad proteins during chondrogenesis and hypertrophic inhibition (185). The interplay between biochemical signalling and mechanical changes in the environment was also observed during physical stimulation of human MSCs using low intensity ultrasound: physical stimulation increased TGF-β-mediated chondrogenic differentiation, highlighting the mechanical sensitivity of chondrogenesis (212).

Studies using BMSCs in collagen gels functionalised with PDT-sensitive particles (section 6.3) suggested that both TGF-ß/Smad and mTOR signalling synergistically activate Sox9-mediated chondrogenesis in response to changes in matrix stiffness and PDT-induced ROS activity (18, 194). Here, enhanced chondrogenesis was due to increased Smad2/3 phosphorylation and reduced integrin ß1 expression in response to matrix stiffening from ~2 to 28.7/40 kPa, as well as increased phosphorylation of mTOR following PDT-induced ROS formation (18, 194). Smad2/3-mediated signalling was also involved in the improvement of pig MSC chondrogenesis and suppression of hypertrophy via inhibition of HIF-1α with DMOG in alginate gels; this was further enhanced after co-delivery of BMP-2 and TGF-ß3 *in vivo* (190). This suggests that Smad2/3 activation is favourable for chondrogenesis, whereas Smad3 activation alone leads to HIF-1α-mediated collagen I production (213).

Synergistic TGF-ß3/KGN action was shown to be effective at promoting MSC chondrogenesis in alginate/gelatin gels, where TGF-ß3 could increase Smad3 phosphorylation and KGN attenuated Runx1 degradation (133). Indeed, it has been shown that various Smad proteins interact with Runx1 (214) and that Smad-Runx interactions are TGF-ß-dependent (215). Additionally, Runx1 knockdown has previously been shown to inhibit chondrogenesis (216) and Runx1 is highly expressed during chondrogenesis compared to Runx2, which is instead more implicated in bone development (217). These studies highlight Runx1 as a specific Runx family member that could play a key role in TGF-ß/Smad-mediated chondrogenesis. It is also probable, like in hypoxic environments and during mechanical stress, that Smad2/3 coactivation occurs, rather than just Smad3, in combination with suppression of Smad1/5/8 activity to promote chondrogenesis and inhibit hypertrophy. Additionally, it is worth noting that KGN-mediated Runx1 activity is related to its disruption of Runx1 interactions with core-binding factor ß1 subunit (CBFß) following KGN binding to filamin A (FLNA) (218). Since FLNA is involved in binding and crosslinking actin filaments (219), one can associate KGN-induced chondrogenesis with disruptions in cytoskeletal organisation and dynamics, which is known to induce BMSC chondrogenesis through changes in integrin-mediated adhesion to hydrogels (220).

However, the study showed that KGN treatment did not significantly influence actin organisation (218), which suggests that KGN-FLNA binding regulates Runx1-CRFß-mediated chondrogenesis via an alternative mechanism.

TGF-ß/Smad-mediated stem cell chondrogenesis in hydrogel microenvironments is evidently sensitive to various cues, including matrix stiffness, ROS, dynamic mechanical stimulation, and hypoxia. The specific Smads and expression patterns involved in stem cell chondrogenesis and suppression of hypertrophy are likely to involve high expression of activated p-Smad2/3, with reduced expression and activity of Smad1/5/8, as exemplified in **Figure 9**. There is also evidence to suggest crosstalk and synergy with other types of signalling, such as p38 MAPKs, mTOR, and Runx1, which collectively represent interesting targets for cartilage repair therapies.

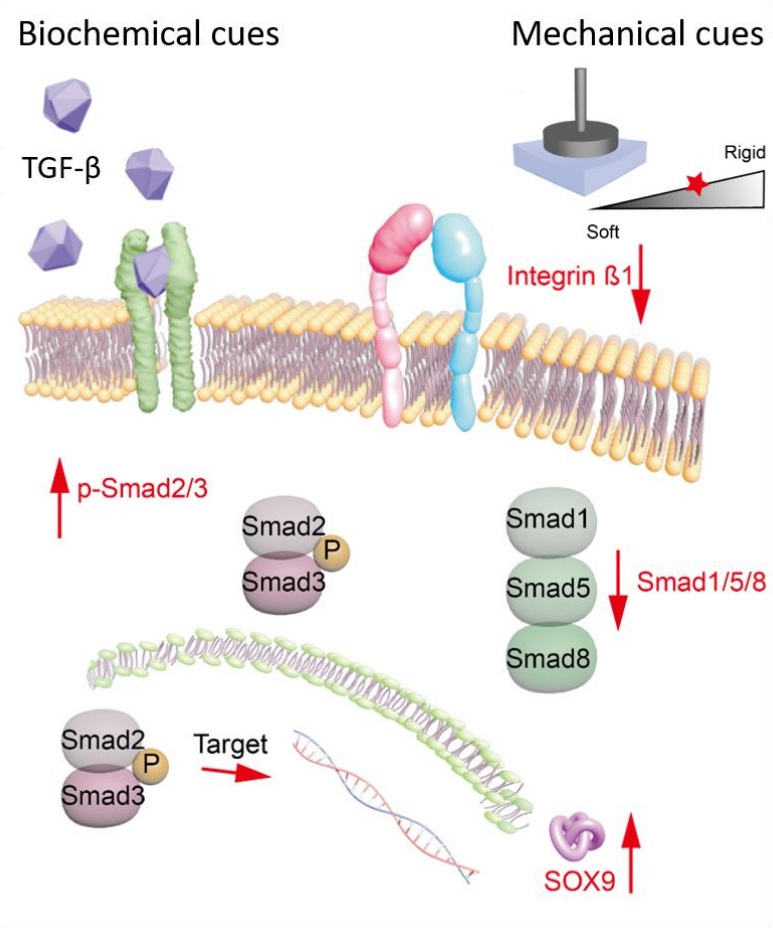

Figure 9. TGF-ß/Smad signalling events that promote hydrogel-regulated stem cell chondrogenesis.

7.2. Wnt/ß-catenin signalling

Wnt signalling was initially identified as a key pathway in regulating embryonic development by controlling processes such as cell fate, proliferation, and migration. Since then, it has also been implicated in tissue regeneration in adult bone marrow, skin, and intestine (221). The canonical Wnt pathway involves cytoplasmic accumulation and nuclear translocation of ß-catenin that facilitates the activation of transcription factor (TCF)/lymphoid enhancer-binding factor (LEF) proteins to activate

target genes. Sox9 is also associated with Wnt/ß-catenin signalling by regulating proliferation and chondrocyte hypertrophy through Sox9 competing with ß-catenin for binding to TCF/LEF sites and the formation of a Sox9:ß-catenin complex which causes the degradation of both proteins (206). It is believed that a delicate balance between Sox9 and ß-catenin is important, where specific relative levels of each are required for controlled chondrocyte differentiation (206). Moreover, Kirton and co-authors found that the activation of canonical Wnt/ß-catenin signalling enhanced pericyte chondrogenesis (222), and Ryu et al. showed that ß-catenin expression was significantly higher in prechondrogenic mesenchymal cells than in differentiated chondrocytes. This suggests that the activation of Wnt signalling is important during early chondrogenic differentiation (223).

Canonical Wnt/ß-catenin-mediated signalling has been implicated in hydrogel-driven chondrogenesis. Indeed, Li et al. (section 2.3.2) found that inhibition of Wnt/ß-catenin signalling and ß-catenin degradation correlated with increased human MSC chondrogenesis in self-assembling KLD-12 peptide gels functionalised with HAVDI (80). This study also showed that while inhibition of Wnt/ß-catenin signalling for 3 days increased chondrogenic gene expression, prolonged inhibition for 14 days inhibited chondrogenesis (80). In agreement with previous work by Tufan and Tuan et al., this suggests that the Wnt inhibitory effect must be downregulated at later stages of chondrogenesis to allow mature chondrogenic differentiation (224). It is also known that N-cadherin hinders canonical Wnt signalling through intracellular interactions with Wnt co-receptor LRP5 which facilitates ß-catenin degradation (225, 226). Several studies have shown that canonical Wnt activation and ß-catenin accumulation negatively regulates chondrogenesis through decreased Sox9 expression, increased expression of anti-chondrogenic gene Twist-1, and impaired transcription of chondrogenic marker genes which limit cartilage regeneration (227-231). The role of ß-catenin is evidently crucial in regulating chondrogenesis where nullification of ß-catenin translocation into the nucleus is important for repression of anti-chondrogenic gene transcription mediated by ß-catenin/TCF/LEF interactions (232, 233). Conversely, Vega et al. (sections 2.3.2 and 5.2) observed that enhanced Wnt/ß-catenin signalling through higher nuclear ß-catenin localisation coincided with increased chondrogenesis of human MSCs after 3 days of culture within methacrylated HA gels functionalised with HAVDI (110); this also correlated with increased Sox9 expression (109). Interestingly, nuclear ß-catenin was most pronounced in cell clusters, compared to single cells, suggesting that cell-cell contacts are important in regulating Wnt/ß-catenin-mediated chondrogenesis (110). Indeed, it has been shown that regulation of ß-catenin phosphorylation is sensitive to cell-cell contacts (234). The observed differences in Wnt/ß-catenin signalling in these hydrogel-based studies could be attributed to the different microenvironment conditions, such as the hydrogel materials used, which may regulate stem cell chondrogenesis via alternative mechanisms. Indeed, Vega et al. showed with HA hydrogels that CD44 inhibition of MSCs decreased chondrogenesis (109), supporting previous studies highlighting the key role of HA-CD44 interactions in chondrogenesis (48, 49). Additionally, it has been shown that changes in HA molecular weight regulates chondrogenesis via the CD44/ERK/Sox9 pathway (235). Collectively, these studies show that as well as cadherin-mimetic peptides like HAVDI, the bioactive

properties of certain materials can also regulate the chondrogenic fate, and may operate through alternative pathways to Wnt/ß-catenin signalling.

Overall, Wnt/ß-catenin signalling is clearly a key regulator of stem cell chondrogenesis, however more investigations are needed to elucidate its role in hydrogel microenvironments (**Figure 10**).

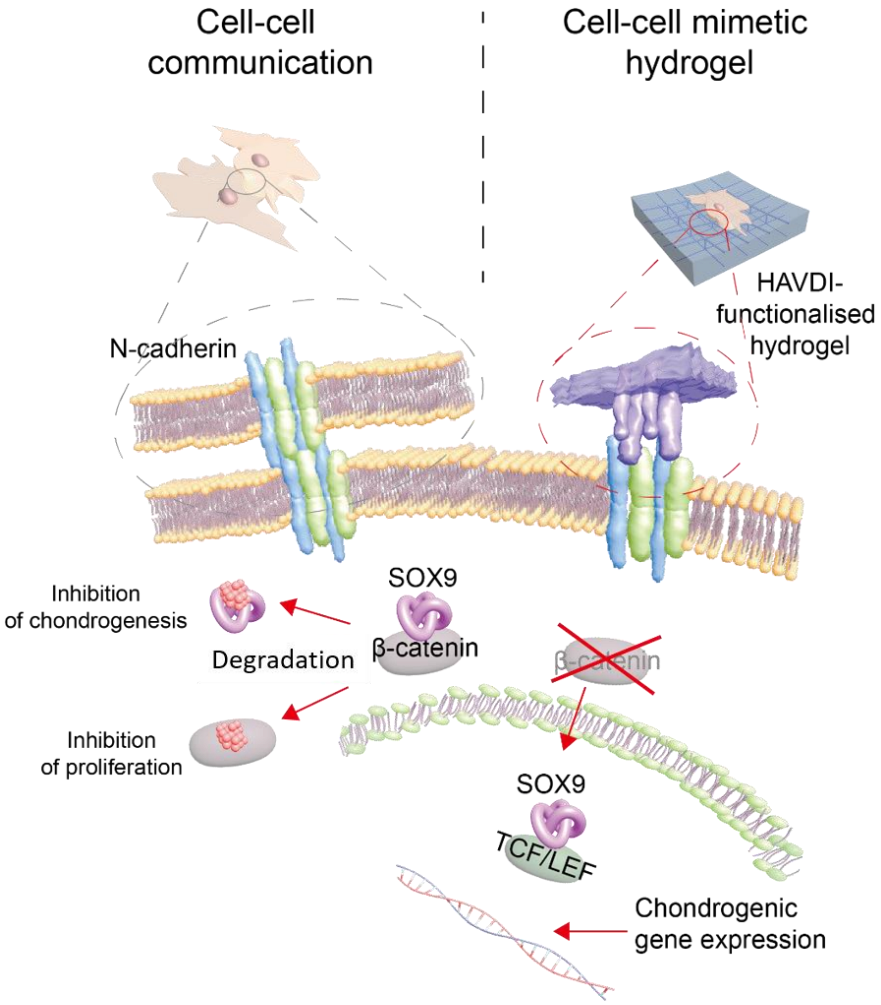

Figure 10. Wnt/ß-catenin signalling mechanisms involved in hydrogel-driven stem cell chondrogenesis.

7.3. ROCK signalling

ROCK is a kinase belonging to the ACG family of serine-threonine kinases and is a major downstream effector of small GTPase Rho. ROCK was first discovered as a Rho effector that induces stress fibre and FA formation through myosin light chain (MLC) phosphorylation and has since been identified as a key cytoskeletal regulator that influences cell morphology and migration (236).

ROCK signalling has been shown to influence stem cell chondrogenesis in hydrogels through changes in cytoskeletal tension. Indeed, while on rigid surfaces ROCK

inhibition via Y-27632 decreased cell spreading and enhanced chondrogenesis (187, 237), on softer hydrogel environments it inhibited chondrogenesis by reducing cytoskeletal tension and increasing spreading (69, 237). This confirms that ROCK activity is fundamental in sensing and responding to ECM stiffness, effectively mediating chondrogenesis in combination with other environmental factors. Moreover, MSC chondrogenic mechanosensitivity to low intensity ultrasound (section 6.1), which was found to be dependent on ERK1/2 phosphorylation, was characterised by a disruption of the actin cytoskeleton similar to the one resulting from ROCK signalling inhibitor Y-27632 (187). This further outlines the role of ERK and ROCK signalling, which have been shown previously to functionally interact (238).

Work in this area suggests that appropriate mechanical environments, where stem cell spreading is restricted, promotes a mesenchymal condensation phenotype that is conducive to chondrogenesis. Further studies into how mechanosensitive ROCK signalling influences stem cell chondrogenesis through cytoskeletal regulation and cell-matrix interactions with hydrogels could highlight its potential as a target for directing cell fate in cartilage engineering (**Figure 11**).

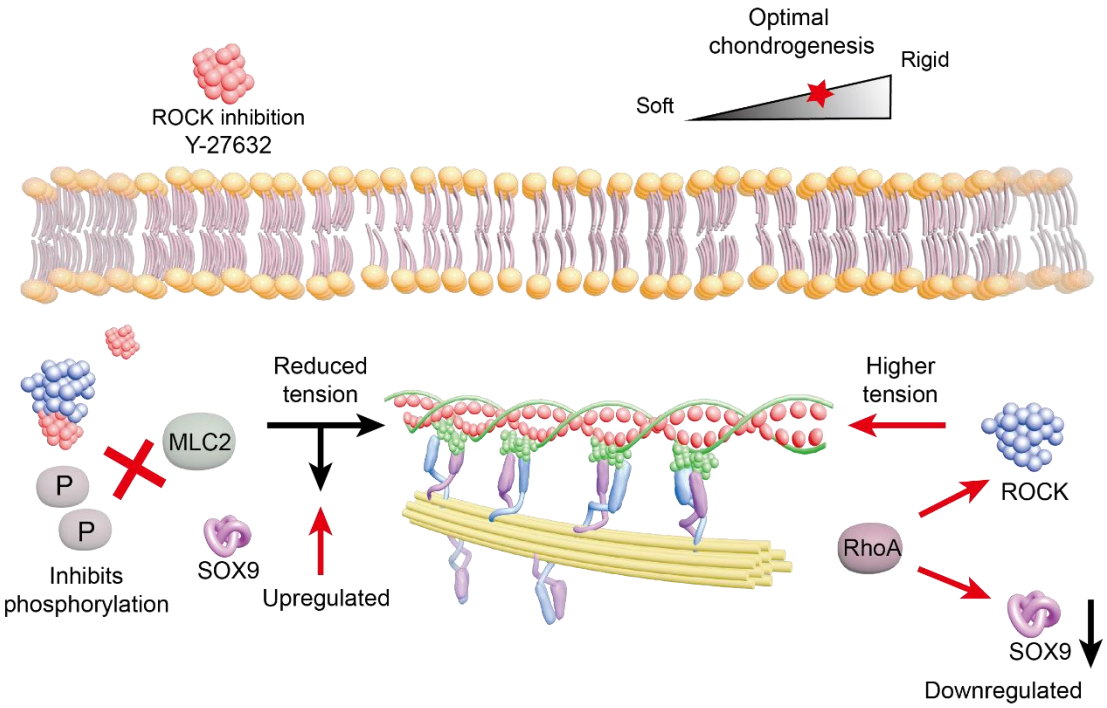

Figure 11. ROCK signalling mechanisms involved in regulating cytoskeletal tension and stem cell chondrogenesis in hydrogel environments.

8. Conclusions and Outlook

Hydrogels have great potential to overcome many of the challenges that the engineering of damaged cartilage tissue still encounters. Indeed, their properties are readily tuneable, and they can be fabricated from a variety of synthetic and natural

materials to facilitate chondrogenesis, effectively harnessing the chondrogenic potential of stem cells. Hydrogels can be designed to be high-strength, mimicking native cartilage properties to withstand physiological mechanical stresses, and can be injectable, allowing for minimally invasive point-of-care treatments and for the bioprinting of structures tailored to meet specific clinical needs. Most importantly, they can be efficiently engineered to recapitulate various properties of native ECMs and to act in synergy with specific external cues, ultimately targeting specific chondrogenic signalling pathways that promote stem cell differentiation into chondrocytes and the generation of a functional cartilage tissue.

The studies reviewed here highlight the plethora of alternative approaches that have been taken to modify the hydrogel properties and to create an environment conducive to stem cell chondrogenesis. The advantages and disadvantages of using specific materials have been addressed to outline some of the key considerations to take when developing chondrogenic hydrogels. It is often ideal to combine different materials and take advantage of their properties in a hybrid hydrogel system, which many studies reported here have achieved successfully. As well as the materials themselves, which may have some bioactive properties, the mechanical and microstructural properties of the hydrogels are also heavily involved in the regulation of chondrogenic fate. While there are no universal optimal conditions for each one of the analysed properties, it is evident that they act collectively in synergy to regulate cell response. Indeed, several researchers have highlighted the mechanosensitivity of various chondrogenic pathways and how standard biochemical cues can be reinforced by physical cues, including hydrogel stiffness or external stimulation. Viscous interactions, whose crucial role in mechanotransduction has been recently established, are also emerging as a potential key regulator of stem cell chondrogenic fate. Another crucial aspect not to be overlooked is the cell population that is encapsulated within the hydrogels: cells' sensing of their surroundings depends as much from their neighbouring cells as it does from the physicochemical properties of the environment itself. As such, cell-cell communication is key in regulating their overall chondrogenic fate. Collectively, all the hydrogel properties and cues discussed in this review converge to regulate key chondrogenesis signalling pathways, including TGF-ß/Smad, Wnt/ß-catenin and ROCK. This provides scope for directing the chondrogenic fate by manipulation of the specific signalling events involved, via modification of cues of the hydrogel microenvironment that control these events in a mechano- and biochemical-sensitive manner. Strikingly, the role of the mechanosensitive YAP/TAZ pathway in hydrogel-driven chondrogenesis remains relatively unexplored, considering that it is regarded as a mechanical rheostat (239) fundamental in cell's response to mechanical cues, and that YAP has been identified as a negative regulator of chondrogenesis in MSCs (240, 241). Elucidating the role of this signalling pathway might reveal cross-talks with other established chondrogenic cues that can be targeted via hydrogel engineering. Critically, the knowledge of the pathways involved in chondrogenic differentiation should guide hydrogel design, and systematic studies in this sense are needed.

Going forward, some of the major challenges in developing hydrogels for cartilage engineering lie in the design of scaffolds with collective optimal properties, including biocompatibility, biodegradability, mechanical stability, and injectability, while reducing

costs. Within this respect, the use of hydrogels functionalised with mimetic peptides might provide a suitable alternative to the use of full-length growth factors. Another issue which often hinders cartilage engineering is the requirement of sourcing cells at densities that are sufficiently high for effective *in vivo* cartilage formation. In this regard, the development of efficient acellular hydrogels or of hydrogel systems that facilitate effective chondrogenesis at lower cell densities is highly desirable. Ultimately, integrating the merits of biomaterials to develop cost-effective hydrogels that can facilitate stem cell chondrogenesis and be administered effectively with minimal invasion and cell content will be critical for their clinical application in cartilage engineering.

Acknowledgements

This work was funded by a grant from the UK Regenerative Medicine Platform. MC acknowledges MRC funding (MR/S005412/1).

References

1. Sophia Fox AJ, Bedi A, Rodeo SA. The basic science of articular cartilage: structure, composition, and function. Sports Health. 2009;1(6):461-8.
2. Sun AX, Lin H, Fritch MR, Shen H, Alexander PG, DeHart M, et al. Chondrogenesis of human bone marrow mesenchymal stem cells in 3-dimensional, photocrosslinked hydrogel constructs: Effect of cell seeding density and material stiffness. Acta Biomater. 2017;58:302-11.
3. Yamzon JL, Kokorowski P, Koh CJ. Stem cells and tissue engineering applications of the genitourinary tract. Pediatr Res. 2008;63(5):472-7.
4. Park MH, Subbiah R, Kwon MJ, Kim WJ, Kim SH, Park K, et al. The three dimensional cues-integrated-biomaterial potentiates differentiation of human mesenchymal stem cells. Carbohydr Polym. 2018;202:488-96.
5. Chen J, Chin A, Almarza AJ, Taboas JM. Hydrogel to guide chondrogenesis versus osteogenesis of mesenchymal stem cells for fabrication of cartilaginous tissues. Biomed Mater. 2020;15(4):045006.
6. Chung C, Burdick JA. Influence of three-dimensional hyaluronic acid microenvironments on mesenchymal stem cell chondrogenesis. Tissue Eng Part A. 2009;15(2):243-54.
7. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. Cell. 2006;126(4):677-89.
8. Bachmann B, Spitz S, Schadl B, Teuschl AH, Redl H, Nurnberger S, et al. Stiffness Matters: Fine-Tuned Hydrogel Elasticity Alters Chondrogenic Redifferentiation. Front Bioeng Biotechnol. 2020;8:373.
9. Li L, Yu F, Zheng L, Wang R, Yan W, Wang Z, et al. Natural hydrogels for cartilage regeneration: Modification, preparation and application. J Orthop Translat. 2019;17:26-41.
10. Yang J, Zhang YS, Yue K, Khademhosseini A. Cell-laden hydrogels for osteochondral and cartilage tissue engineering. Acta Biomater. 2017;57:1-25.

11. Li J, Chen G, Xu X, Abdou P, Jiang Q, Shi D, et al. Advances of injectable hydrogel-based scaffolds for cartilage regeneration. Regen Biomater. 2019;6(3):129-40.
12. Rim YA, Nam Y, Park N, Lee J, Park SH, Ju JH. Repair potential of nonsurgically delivered induced pluripotent stem cell-derived chondrocytes in a rat osteochondral defect model. J Tissue Eng Regen Med. 2018;12(8):1843-55.
13. Caron MM, Emans PJ, Coolsen MM, Voss L, Surtel DA, Cremers A, et al. Redifferentiation of dedifferentiated human articular chondrocytes: comparison of 2D and 3D cultures. Osteoarthritis Cartilage. 2012;20(10):1170-8.
14. Boeuf S, Richter W. Chondrogenesis of mesenchymal stem cells: role of tissue source and inducing factors. Stem Cell Res Ther. 2010;1(4):31.
15. Woods A, Wang G, Beier F. Regulation of chondrocyte differentiation by the actin cytoskeleton and adhesive interactions. J Cell Physiol. 2007;213(1):1-8.
16. Parmar PA, Skaalure SC, Chow LW, St-Pierre JP, Stoichevska V, Peng YY, et al. Temporally degradable collagen-mimetic hydrogels tuned to chondrogenesis of human mesenchymal stem cells. Biomaterials. 2016;99:56-71.
17. Yang J, Li Y, Liu Y, Li D, Zhang L, Wang Q, et al. Influence of hydrogel network microstructures on mesenchymal stem cell chondrogenesis in vitro and in vivo. Acta Biomater. 2019;91:159-72.
18. Zheng L, Liu S, Cheng X, Qin Z, Lu Z, Zhang K, et al. Intensified Stiffness and Photodynamic Provocation in a Collagen-Based Composite Hydrogel Drive Chondrogenesis. Adv Sci (Weinh). 2019;6(16):1900099.
19. Yang J, Xiao Y, Tang Z, Luo Z, Li D, Wang Q, et al. The negatively charged microenvironment of collagen hydrogels regulates the chondrogenic differentiation of bone marrow mesenchymal stem cells in vitro and in vivo. J Mater Chem B. 2020;8(21):4680-93.
20. Jiang T, Liu J, Ouyang Y, Wu H, Zheng L, Zhao J, et al. Intra-hydrogel culture prevents transformation of mesenchymal stem cells induced by monolayer expansion. Biomater Sci. 2018;6(5):1168-76.
21. Vazquez-Portalati NN, Kilmer CE, Panitch A, Liu JC. Characterization of Collagen Type I and II Blended Hydrogels for Articular Cartilage Tissue Engineering. Biomacromolecules. 2016;17(10):3145-52.
22. Courtenay JS, Dallman MJ, Dayan AD, Martin A, Mosedale B. Immunisation against heterologous type II collagen induces arthritis in mice. Nature. 1980;283(5748):666-8.
23. Yuan L, Li B, Yang J, Ni Y, Teng Y, Guo L, et al. Effects of Composition and Mechanical Property of Injectable Collagen I/II Composite Hydrogels on Chondrocyte Behaviors. Tissue Eng Part A. 2016;22(11-12):899-906.
24. Sarem M, Arya N, Heizmann M, Neffe AT, Barbero A, Gebauer TP, et al. Interplay between stiffness and degradation of architectured gelatin hydrogels leads to differential modulation of chondrogenesis in vitro and in vivo. Acta Biomater. 2018;69:83-94.
25. Zigon-Branc S, Markovic M, Van Hoorick J, Van Vlierberghe S, Dubruel P, Zerobin E, et al. Impact of Hydrogel Stiffness on Differentiation of Human Adipose-Derived Stem Cell Microspheroids. Tissue Eng Part A. 2019;25(19-20):1369-80.
26. Moulisova V, Poveda-Reyes S, Sanmartin-Masia E, Quintanilla-Sierra L, Salmeron-Sanchez M, Gallego Ferrer G. Hybrid Protein-Glycosaminoglycan Hydrogels Promote Chondrogenic Stem Cell Differentiation. ACS Omega. 2017;2(11):7609-20.

27. Gornall JL, Terentjev EM. Helix-coil transition of gelatin: helical morphology and stability. Soft Matter. 2008;4(3):544-9.
28. Harrington WF, Rao NV. Collagen structure in solution. I. Kinetics of helix regeneration in single-chain gelatins. Biochemistry. 1970;9(19):3714-24.
29. Francis SL, Di Bella C, Wallace GG, Choong PFM. Cartilage Tissue Engineering Using Stem Cells and Bioprinting Technology-Barriers to Clinical Translation. Front Surg. 2018;5:70.
30. Swinehart IT, Badylak SF. Extracellular matrix bioscaffolds in tissue remodeling and morphogenesis. Dev Dyn. 2016;245(3):351-60.
31. Gilchrist AE, Lee S, Hu Y, Harley BAC. Soluble Signals and Remodeling in a Synthetic Gelatin-Based Hematopoietic Stem Cell Niche. Adv Healthc Mater. 2019;8(20):e1900751.
32. Han S, Li YY, Chan BP. Extracellular Protease Inhibition Alters the Phenotype of Chondrogenically Differentiating Human Mesenchymal Stem Cells (MSCs) in 3D Collagen Microspheres. PLoS One. 2016;11(1):e0146928.
33. Radhakrishnan J, Subramanian A, Krishnan UM, Sethuraman S. Injectable and 3D Bioprinted Polysaccharide Hydrogels: From Cartilage to Osteochondral Tissue Engineering. Biomacromolecules. 2017;18(1):1-26.
34. Jahanbakhsh A, Nourbakhsh MS, Bonakdar S, Shokrgozar MA, Haghighipour N. Evaluation of alginate modification effect on cell-matrix interaction, mechanotransduction and chondrogenesis of encapsulated MSCs. Cell Tissue Res. 2020;381(2):255-72.
35. Pustlauk W, Paul B, Gelinsky M, Bernhardt A. Jellyfish collagen and alginate: Combined marine materials for superior chondrogenesis of hMSC. Mater Sci Eng C Mater Biol Appl. 2016;64:190-8.
36. Karunanithi P, Murali MR, Samuel S, Raghavendran HRB, Abbas AA, Kamarul T. Three dimensional alginate-fucoidan composite hydrogel augments the chondrogenic differentiation of mesenchymal stromal cells. Carbohydr Polym. 2016;147:294-303.
37. Maturavongsadit P, Bi X, Metavarayuth K, Luckanagul JA, Wang Q. Influence of Cross-Linkers on the in Vitro Chondrogenesis of Mesenchymal Stem Cells in Hyaluronic Acid Hydrogels. ACS Appl Mater Interfaces. 2017;9(4):3318-29.
38. Kwon MY, Vega SL, Gramlich WM, Kim M, Mauck RL, Burdick JA. Dose and Timing of N-Cadherin Mimetic Peptides Regulate MSC Chondrogenesis within Hydrogels. Adv Healthc Mater. 2018;7(9):e1701199.
39. Baeurle S, Kiselev, M., Makarova, E. and Nogovitsin, E. Effect of the counterion behavior on the frictional–compressive properties of chondroitin sulfate solutions. Polymer. 2009;50(7):1805-13.
40. Rich AM, Pearlstein E, Weissmann G, Hoffstein ST. Cartilage proteoglycans inhibit fibronectin-mediated adhesion. Nature. 1981;293(5829):224-6.
41. Knox P, Wells P. Cell adhesion and proteoglycans. I. The effect of exogenous proteoglycans on the attachment of chick embryo fibroblasts to tissue culture plastic and collagen. J Cell Sci. 1979;40:77-88.
42. Yamagata M, Suzuki S, Akiyama SK, Yamada KM, Kimata K. Regulation of cell-substrate adhesion by proteoglycans immobilized on extracellular substrates. J Biol Chem. 1989;264(14):8012-8.
43. Lim HC, Multhaupt HA, Couchman JR. Cell surface heparan sulfate proteoglycans control adhesion and invasion of breast carcinoma cells. Mol Cancer. 2015;14:15.

44. Stichler S, Bock T, Paxton N, Bertlein S, Levato R, Schill V, et al. Double printing of hyaluronic acid/poly(glycidol) hybrid hydrogels with poly(epsilon-caprolactone) for MSC chondrogenesis. Biofabrication. 2017;9(4):044108.
45. Ansari S, Diniz IM, Chen C, Aghaloo T, Wu BM, Shi S, et al. Alginate/hyaluronic acid hydrogel delivery system characteristics regulate the differentiation of periodontal ligament stem cells toward chondrogenic lineage. J Mater Sci Mater Med. 2017;28(10):162.
46. Wang CZ, Eswaramoorthy R, Lin TH, Chen CH, Fu YC, Wang CK, et al. Enhancement of chondrogenesis of adipose-derived stem cells in HA-PNIPAAm-CL hydrogel for cartilage regeneration in rabbits. Sci Rep. 2018;8(1):10526.
47. Costantini M, Idaszek J, Szoke K, Jaroszewicz J, Dentini M, Barbetta A, et al. 3D bioprinting of BM-MSCs-loaded ECM biomimetic hydrogels for in vitro neocartilage formation. Biofabrication. 2016;8(3):035002.
48. Kwon MY, Wang C, Galarraga JH, Pure E, Han L, Burdick JA. Influence of hyaluronic acid modification on CD44 binding towards the design of hydrogel biomaterials. Biomaterials. 2019;222:119451.
49. Wu SC, Chen CH, Chang JK, Fu YC, Wang CK, Eswaramoorthy R, et al. Hyaluronan initiates chondrogenesis mainly via CD44 in human adipose-derived stem cells. J Appl Physiol (1985). 2013;114(11):1610-8.
50. Wang T, Yang F. A comparative study of chondroitin sulfate and heparan sulfate for directing three-dimensional chondrogenesis of mesenchymal stem cells. Stem Cell Res Ther. 2017;8(1):284.
51. Wang T, Lai JH, Yang F. Effects of Hydrogel Stiffness and Extracellular Compositions on Modulating Cartilage Regeneration by Mixed Populations of Stem Cells and Chondrocytes In Vivo. Tissue Eng Part A. 2016;22(23-24):1348-56.
52. Burnsed OA, Schwartz Z, Marchand KO, Hyzy SL, Olivares-Navarrete R, Boyan BD. Hydrogels derived from cartilage matrices promote induction of human mesenchymal stem cell chondrogenic differentiation. Acta Biomater. 2016;43:139-49.
53. Beck EC, Barragan M, Tadros MH, Gehrke SH, Detamore MS. Approaching the compressive modulus of articular cartilage with a decellularized cartilage-based hydrogel. Acta Biomater. 2016;38:94-105.
54. Lindberg GCJ, Longoni A, Lim KS, Rosenberg AJ, Hooper GJ, Gawlitta D, et al. Intact vitreous humor as a potential extracellular matrix hydrogel for cartilage tissue engineering applications. Acta Biomater. 2019;85:117-30.
55. Jooybar E, Abdekhodaie MJ, Alvi M, Mousavi A, Karperien M, Dijkstra PJ. An injectable platelet lysate-hyaluronic acid hydrogel supports cellular activities and induces chondrogenesis of encapsulated mesenchymal stem cells. Acta Biomater. 2019;83:233-44.
56. Gao X, Gao L, Groth T, Liu T, He D, Wang M, et al. Fabrication and properties of an injectable sodium alginate/PRP composite hydrogel as a potential cell carrier for cartilage repair. J Biomed Mater Res A. 2019;107(9):2076-87.
57. Rothrauff BB, Shimomura K, Gottardi R, Alexander PG, Tuan RS. Anatomical region-dependent enhancement of 3-dimensional chondrogenic differentiation of human mesenchymal stem cells by soluble meniscus extracellular matrix. Acta Biomater. 2017;49:140-51.
58. Almeida HV, Eswaramoorthy R, Cunniffe GM, Buckley CT, O'Brien FJ, Kelly DJ. Fibrin hydrogels functionalized with cartilage extracellular matrix and incorporating freshly isolated stromal cells as an injectable for cartilage regeneration. Acta Biomater. 2016;36:55-62.

59. Liou JJ, Rothrauff BB, Alexander PG, Tuan RS. Effect of Platelet-Rich Plasma on Chondrogenic Differentiation of Adipose- and Bone Marrow-Derived Mesenchymal Stem Cells. Tissue Eng Part A. 2018;24(19-20):1432-43.
60. Beck EC, Barragan M, Tadros MH, Kiyotake EA, Acosta FM, Kieweg SL, et al. Chondroinductive Hydrogel Pastes Composed of Naturally Derived Devitalized Cartilage. Ann Biomed Eng. 2016;44(6):1863-80.
61. Kim JS, Kim TH, Kang DL, Baek SY, Lee Y, Koh YG, et al. Chondrogenic differentiation of human ASCs by stiffness control in 3D fibrin hydrogel. Biochem Biophys Res Commun. 2020;522(1):213-9.
62. Yang R, Wang X, Liu S, Zhang W, Wang P, Liu X, et al. Bioinspired poly (gamma-glutamic acid) hydrogels for enhanced chondrogenesis of bone marrow-derived mesenchymal stem cells. Int J Biol Macromol. 2020;142:332-44.
63. Chen H, Wang, H., Li, B., Feng, B., He, X., Fu, W., Yuan, H. and Xu, Z. Enhanced chondrogenic differentiation of human mesenchymal stems cells on citric acid-modified chitosan hydrogel for tracheal cartilage regeneration applications. RSC Advances. 2018;8(30):16910-7.
64. Zhu J, Marchant RE. Design properties of hydrogel tissue-engineering scaffolds. Expert Rev Med Devices. 2011;8(5):607-26.
65. Guan X, Avci-Adali M, Alarcin E, Cheng H, Kashaf SS, Li Y, et al. Development of hydrogels for regenerative engineering. Biotechnol J. 2017;12(5).
66. Carrion B, Souzanchi MF, Wang VT, Tiruchinapally G, Shikanov A, Putnam AJ, et al. The Synergistic Effects of Matrix Stiffness and Composition on the Response of Chondroprogenitor Cells in a 3D Precondensation Microenvironment. Adv Healthc Mater. 2016;5(10):1192-202.
67. Shen H, Lin H, Sun AX, Song S, Zhang Z, Dai J, et al. Chondroinductive factor-free chondrogenic differentiation of human mesenchymal stem cells in graphene oxide-incorporated hydrogels. J Mater Chem B. 2018;6(6):908-17.
68. Caliari SR, Burdick JA. A practical guide to hydrogels for cell culture. Nat Methods. 2016;13(5):405-14.
69. Foyt DA, Taheem DK, Ferreira SA, Norman MDA, Petzold J, Jell G, et al. Hypoxia impacts human MSC response to substrate stiffness during chondrogenic differentiation. Acta Biomater. 2019;89:73-83.
70. Nagase K, Yamato M, Kanazawa H, Okano T. Poly(N-isopropylacrylamide)-based thermoresponsive surfaces provide new types of biomedical applications. Biomaterials. 2018;153:27-48.
71. Lanzalaco S, Armelin E. Poly(N-isopropylacrylamide) and Copolymers: A Review on Recent Progresses in Biomedical Applications. Gels. 2017;3(4).
72. Gong J, Katsuyama, Y., Kurokawa, T. and Osada, Y. Double-Network Hydrogels with Extremely High Mechanical Strength. Advanced Materials. 2003;15(14):1155-8.
73. Liu Y, Zhang K, Ma J, Vancso GJ. Thermoresponsive Semi-IPN Hydrogel Microfibers from Continuous Fluidic Processing with High Elasticity and Fast Actuation. ACS Appl Mater Interfaces. 2017;9(1):901-8.
74. Petrusic S, Lewandowski, M., Giraud, S., Jovancic, P., Bugarski, B., Ostojic, S. and Koncar, V. Development and characterization of thermosensitive hydrogels based on poly(N-isopropylacrylamide) and calcium alginate. Journal of Applied Polymer Science. 2011;124(2):890-903.
75. Brunelle AR, Horner CB, Low K, Ico G, Nam J. Electrospun thermosensitive hydrogel scaffold for enhanced chondrogenesis of human mesenchymal stem cells. Acta Biomater. 2018;66:166-76.

76. Mellati A, Fan CM, Tamayol A, Annabi N, Dai S, Bi J, et al. Microengineered 3D cell-laden thermoresponsive hydrogels for mimicking cell morphology and orientation in cartilage tissue engineering. Biotechnol Bioeng. 2017;114(1):217-31.
77. Xu G, Ding Z, Lu Q, Zhang X, Zhou X, Xiao L, et al. Electric field-driven building blocks for introducing multiple gradients to hydrogels. Protein Cell. 2020;11(4):267-85.
78. Kisiday JD, Colbath AC, Tangtrongsup S. Effect of culture duration on chondrogenic preconditioning of equine bone marrow mesenchymal stem cells in self-assembling peptide hydrogel. J Orthop Res. 2019;37(6):1368-75.
79. Lu J, Shen X, Sun X, Yin H, Yang S, Lu C, et al. Increased recruitment of endogenous stem cells and chondrogenic differentiation by a composite scaffold containing bone marrow homing peptide for cartilage regeneration. Theranostics. 2018;8(18):5039-58.
80. Li R, Xu J, Wong DSH, Li J, Zhao P, Bian L. Self-assembled N-cadherin mimetic peptide hydrogels promote the chondrogenesis of mesenchymal stem cells through inhibition of canonical Wnt/beta-catenin signaling. Biomaterials. 2017;145:33-43.
81. Ren K, Cui H, Xu Q, He C, Li G, Chen X. Injectable Polypeptide Hydrogels with Tunable Microenvironment for 3D Spreading and Chondrogenic Differentiation of Bone-Marrow-Derived Mesenchymal Stem Cells. Biomacromolecules. 2016;17(12):3862-71.
82. Lee SS, Choi GE, Lee HJ, Kim Y, Choy JH, Jeong B. Layered Double Hydroxide and Polypeptide Thermogel Nanocomposite System for Chondrogenic Differentiation of Stem Cells. ACS Appl Mater Interfaces. 2017;9(49):42668-75.
83. Parmar PA, St-Pierre JP, Chow LW, Spicer CD, Stoichevska V, Peng YY, et al. Enhanced articular cartilage by human mesenchymal stem cells in enzymatically mediated transiently RGDS-functionalized collagen-mimetic hydrogels. Acta Biomater. 2017;51:75-88.
84. Chen J, Zou X. Self-assemble peptide biomaterials and their biomedical applications. Bioact Mater. 2019;4:120-31.
85. Liu C, Zhang, Q., Zhu, S., Liu, H. and Chen, J. Preparation and applications of peptide-based injectable hydrogels. RCS Advances. 2019;9(48):28299-311.
86. Liu Q, Jia Z, Duan L, Xiong J, Wang D, Ding Y. Functional peptides for cartilage repair and regeneration. Am J Transl Res. 2018;10(2):501-10.
87. Mahzoon S, Townsend JM, Lam TN, Sjoelund V, Detamore MS. Effects of a Bioactive SPPEPS Peptide on Chondrogenic Differentiation of Mesenchymal Stem Cells. Ann Biomed Eng. 2019;47(11):2308-21.
88. Park SS, J. Park, J. Ji, Y. Kim, K. Choi, H. Choi, S. Kim, J. Min, B. Kim, M. An injectable, click-crosslinked, cytomodulin-modified hyaluronic acid hydrogel for cartilage tissue engineering. NPG Asia Materials. 2019;11(1).
89. Pankov R, Yamada KM. Fibronectin at a glance. J Cell Sci. 2002;115(Pt 20):3861-3.
90. Pierschbacher MD, Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature. 1984;309(5963):30-3.
91. Kudva AK, Luyten FP, Patterson J. RGD-functionalized polyethylene glycol hydrogels support proliferation and in vitro chondrogenesis of human periosteum-derived cells. J Biomed Mater Res A. 2018;106(1):33-42.
92. Parmar PA, Chow LW, St-Pierre JP, Horejs CM, Peng YY, Werkmeister JA, et al. Collagen-mimetic peptide-modifiable hydrogels for articular cartilage regeneration. Biomaterials. 2015;54:213-25.

93. DeLise AM, Fischer L, Tuan RS. Cellular interactions and signaling in cartilage development. Osteoarthritis Cartilage. 2000;8(5):309-34.
94. Pfander D, Rahmanzadeh R, Scheller EE. Presence and distribution of collagen II, collagen I, fibronectin, and tenascin in rabbit normal and osteoarthritic cartilage. J Rheumatol. 1999;26(2):386-94.
95. Re'em T, Tsur-Gang O, Cohen S. The effect of immobilized RGD peptide in macroporous alginate scaffolds on TGFbeta1-induced chondrogenesis of human mesenchymal stem cells. Biomaterials. 2010;31(26):6746-55.
96. Nuttelman CR, Tripodi MC, Anseth KS. Synthetic hydrogel niches that promote hMSC viability. Matrix Biol. 2005;24(3):208-18.
97. Ra HJ, Harju-Baker S, Zhang F, Linhardt RJ, Wilson CL, Parks WC. Control of promatrilysin (MMP7) activation and substrate-specific activity by sulfated glycosaminoglycans. J Biol Chem. 2009;284(41):27924-32.
98. Cosgriff-Hernandez E, Hahn MS, Russell B, Wilems T, Munoz-Pinto D, Browning MB, et al. Bioactive hydrogels based on Designer Collagens. Acta Biomater. 2010;6(10):3969-77.
99. Peng YY, Yoshizumi A, Danon SJ, Glattauer V, Prokopenko O, Mirochnitchenko O, et al. A Streptococcus pyogenes derived collagen-like protein as a non-cytotoxic and non-immunogenic cross-linkable biomaterial. Biomaterials. 2010;31(10):2755-61.
100. Parmar PA, St-Pierre JP, Chow LW, Puetzer JL, Stoichevska V, Peng YY, et al. Harnessing the Versatility of Bacterial Collagen to Improve the Chondrogenic Potential of Porous Collagen Scaffolds. Adv Healthc Mater. 2016;5(13):1656-66.
101. Peng YY, Howell L, Stoichevska V, Werkmeister JA, Dumsday GJ, Ramshaw JA. Towards scalable production of a collagen-like protein from Streptococcus pyogenes for biomedical applications. Microb Cell Fact. 2012;11:146.
102. Zhan X. Effect of matrix stiffness and adhesion ligand density on chondrogenic differentiation of mesenchymal stem cells. J Biomed Mater Res A. 2020;108(3):675-83.
103. Vega SL, Kwon MY, Song KH, Wang C, Mauck RL, Han L, et al. Combinatorial hydrogels with biochemical gradients for screening 3D cellular microenvironments. Nat Commun. 2018;9(1):614.
104. Takeichi M. Morphogenetic roles of classic cadherins. Curr Opin Cell Biol. 1995;7(5):619-27.
105. Koch AW, Bozic D, Pertz O, Engel J. Homophilic adhesion by cadherins. Curr Opin Struct Biol. 1999;9(2):275-81.
106. Williams E, Williams G, Gour BJ, Blaschuk OW, Doherty P. A novel family of cyclic peptide antagonists suggests that N-cadherin specificity is determined by amino acids that flank the HAV motif. J Biol Chem. 2000;275(6):4007-12.
107. Singh P, Schwarzbauer JE. Fibronectin matrix assembly is essential for cell condensation during chondrogenesis. J Cell Sci. 2014;127(Pt 20):4420-8.
108. Lee JW, An H, Lee KY. Introduction of N-cadherin-binding motif to alginate hydrogels for controlled stem cell differentiation. Colloids Surf B Biointerfaces. 2017;155:229-37.
109. Bian L, Guvendiren M, Mauck RL, Burdick JA. Hydrogels that mimic developmentally relevant matrix and N-cadherin interactions enhance MSC chondrogenesis. Proc Natl Acad Sci U S A. 2013;110(25):10117-22.
110. Vega SL, Kwon M, Mauck RL, Burdick JA. Single Cell Imaging to Probe Mesenchymal Stem Cell N-Cadherin Mediated Signaling within Hydrogels. Ann Biomed Eng. 2016;44(6):1921-30.

111. Sieminski AL, Semino CE, Gong H, Kamm RD. Primary sequence of ionic self-assembling peptide gels affects endothelial cell adhesion and capillary morphogenesis. J Biomed Mater Res A. 2008;87(2):494-504.
112. Miller RE, Grodzinsky AJ, Vanderploeg EJ, Lee C, Ferris DJ, Barrett MF, et al. Effect of self-assembling peptide, chondrogenic factors, and bone marrow-derived stromal cells on osteochondral repair. Osteoarthritis Cartilage. 2010;18(12):1608-19.
113. Tripathi JK, Pal S, Awasthi B, Kumar A, Tandon A, Mitra K, et al. Variants of self-assembling peptide, KLD-12 that show both rapid fracture healing and antimicrobial properties. Biomaterials. 2015;56:92-103.
114. Zhang S. Fabrication of novel biomaterials through molecular self-assembly. Nat Biotechnol. 2003;21(10):1171-8.
115. Kim SJ, Kim JE, Kim SH, Kim SJ, Jeon SJ, Kim SH, et al. Therapeutic effects of neuropeptide substance P coupled with self-assembled peptide nanofibers on the progression of osteoarthritis in a rat model. Biomaterials. 2016;74:119-30.
116. Shah RN, Shah NA, Del Rosario Lim MM, Hsieh C, Nuber G, Stupp SI. Supramolecular design of self-assembling nanofibers for cartilage regeneration. Proc Natl Acad Sci U S A. 2010;107(8):3293-8.
117. Kisiday JD, Kopesky PW, Evans CH, Grodzinsky AJ, McIlwraith CW, Frisbie DD. Evaluation of adult equine bone marrow- and adipose-derived progenitor cell chondrogenesis in hydrogel cultures. J Orthop Res. 2008;26(3):322-31.
118. He B, Yuan X, Zhou A, Zhang H, Jiang D. Designer functionalised self-assembling peptide nanofibre scaffolds for cartilage tissue engineering. Expert Rev Mol Med. 2014;16:e12.
119. Liu C, Zhu C, Li J, Zhou P, Chen M, Yang H, et al. The effect of the fibre orientation of electrospun scaffolds on the matrix production of rabbit annulus fibrosus-derived stem cells. Bone Res. 2015;3:15012.
120. Guo JL, Li A, Kim YS, Xie VY, Smith BT, Watson E, et al. Click functionalized, tissue-specific hydrogels for osteochondral tissue engineering. J Biomed Mater Res A. 2020;108(3):684-93.
121. Danisovic L, Varga I, Polak S. Growth factors and chondrogenic differentiation of mesenchymal stem cells. Tissue Cell. 2012;44(2):69-73.
122. Rice JJ, Martino MM, De Laporte L, Tortelli F, Briquez PS, Hubbell JA. Engineering the regenerative microenvironment with biomaterials. Adv Healthc Mater. 2013;2(1):57-71.
123. Shen H, Lin H, Sun AX, Song S, Wang B, Yang Y, et al. Acceleration of chondrogenic differentiation of human mesenchymal stem cells by sustained growth factor release in 3D graphene oxide incorporated hydrogels. Acta Biomater. 2020;105:44-55.
124. Toole BP. Hyaluronan and its binding proteins, the hyaladherins. Curr Opin Cell Biol. 1990;2(5):839-44.
125. Deng Y, Sun AX, Overholt KJ, Yu GZ, Fritch MR, Alexander PG, et al. Enhancing chondrogenesis and mechanical strength retention in physiologically relevant hydrogels with incorporation of hyaluronic acid and direct loading of TGF-beta. Acta Biomater. 2019;83:167-76.
126. Feng Q, Lin S, Zhang K, Dong C, Wu T, Huang H, et al. Sulfated hyaluronic acid hydrogels with retarded degradation and enhanced growth factor retention promote hMSC chondrogenesis and articular cartilage integrity with reduced hypertrophy. Acta Biomater. 2017;53:329-42.
127. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. J Cell Sci. 2010;123(Pt 24):4195-200.

128. Brochu AB, Craig SL, Reichert WM. Self-healing biomaterials. J Biomed Mater Res A. 2011;96(2):492-506.
129. Guvendiren M, Lu, H. and Burdick, J. Shear-thinning hydrogels for biomedical applications. Soft Matter. 2012;8(2):260-72.
130. Wei K, Zhu, M., Sun, Y., Xu, J., Feng, Q., Lin, S., Wu, T., Xu, J., Tian, F., Xia, J., Li, G. and Bian, L. Robust Biopolymeric Supramolecular “Host−Guest Macromer” Hydrogels Reinforced by in Situ Formed Multivalent Nanoclusters for Cartilage Regeneration. Macromolecules. 2016;49(3):866-75.
131. Xu J, Feng Q, Lin S, Yuan W, Li R, Li J, et al. Injectable stem cell-laden supramolecular hydrogels enhance in situ osteochondral regeneration via the sustained co-delivery of hydrophilic and hydrophobic chondrogenic molecules. Biomaterials. 2019;210:51-61.
132. Bock T, Schill V, Krahnke M, Steinert AF, Tessmar J, Blunk T, et al. TGF-beta1-Modified Hyaluronic Acid/Poly(glycidol) Hydrogels for Chondrogenic Differentiation of Human Mesenchymal Stromal Cells. Macromol Biosci. 2018;18(7):e1700390.
133. Fan W, Yuan L, Li J, Wang Z, Chen J, Guo C, et al. Injectable double-crosslinked hydrogels with kartogenin-conjugated polyurethane nano-particles and transforming growth factor beta3 for in-situ cartilage regeneration. Mater Sci Eng C Mater Biol Appl. 2020;110:110705.
134. Dehghan-Baniani D, Chen Y, Wang D, Bagheri R, Solouk A, Wu H. Injectable in situ forming kartogenin-loaded chitosan hydrogel with tunable rheological properties for cartilage tissue engineering. Colloids Surf B Biointerfaces. 2020;192:111059.
135. Mahmoudi Z, Mohammadnejad J, Razavi Bazaz S, Abouei Mehrizi A, Saidijam M, Dinarvand R, et al. Promoted chondrogenesis of hMCSs with controlled release of TGF-beta3 via microfluidics synthesized alginate nanogels. Carbohydr Polym. 2020;229:115551.
136. Ji X, Lei Z, Yuan M, Zhu H, Yuan X, Liu W, et al. Cartilage repair mediated by thermosensitive photocrosslinkable TGFbeta1-loaded GM-HPCH via immunomodulating macrophages, recruiting MSCs and promoting chondrogenesis. Theranostics. 2020;10(6):2872-87.
137. Cox TR, Erler JT. Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. Dis Model Mech. 2011;4(2):165-78.
138. Yang K, Sun J, Guo Z, Yang J, Wei D, Tan Y, et al. Methacrylamide-modified collagen hydrogel with improved anti-actin-mediated matrix contraction behavior. J Mater Chem B. 2018;6(45):7543-55.
139. Teong B, Wu SC, Chang CM, Chen JW, Chen HT, Chen CH, et al. The stiffness of a crosslinked hyaluronan hydrogel affects its chondro-induction activity on hADSCs. J Biomed Mater Res B Appl Biomater. 2018;106(2):808-16.
140. Vega SL, Kwon MY, Burdick JA. Recent advances in hydrogels for cartilage tissue engineering. Eur Cell Mater. 2017;33:59-75.
141. Armstrong CG, Mow VC. Variations in the intrinsic mechanical properties of human articular cartilage with age, degeneration, and water content. J Bone Joint Surg Am. 1982;64(1):88-94.
142. Little CJ, Bawolin NK, Chen X. Mechanical properties of natural cartilage and tissue-engineered constructs. Tissue Eng Part B Rev. 2011;17(4):213-27.
143. Tatman PD, Gerull W, Sweeney-Easter S, Davis JI, Gee AO, Kim DH. Multiscale Biofabrication of Articular Cartilage: Bioinspired and Biomimetic Approaches. Tissue Eng Part B Rev. 2015;21(6):543-59.

144. Oria R, Wiegand T, Escribano J, Elosegui-Artola A, Uriarte JJ, Moreno-Pulido C, et al. Force loading explains spatial sensing of ligands by cells. Nature. 2017;552(7684):219-24.
145. Peyton SR, Putnam AJ. Extracellular matrix rigidity governs smooth muscle cell motility in a biphasic fashion. J Cell Physiol. 2005;204(1):198-209.
146. Shin JW, Mooney DJ. Extracellular matrix stiffness causes systematic variations in proliferation and chemosensitivity in myeloid leukemias. Proc Natl Acad Sci U S A. 2016;113(43):12126-31.
147. Cantini M, Donnelly H, Dalby MJ, Salmeron-Sanchez M. The Plot Thickens: The Emerging Role of Matrix Viscosity in Cell Mechanotransduction. Adv Healthc Mater. 2020;9(8):e1901259.
148. Chaudhuri O. Viscoelastic hydrogels for 3D cell culture. Biomater Sci. 2017;5(8):1480-90.
149. Chaudhuri O, Gu L, Klumpers D, Darnell M, Bencherif SA, Weaver JC, et al. Hydrogels with tunable stress relaxation regulate stem cell fate and activity. Nat Mater. 2016;15(3):326-34.
150. Charrier EE, Pogoda K, Wells RG, Janmey PA. Control of cell morphology and differentiation by substrates with independently tunable elasticity and viscous dissipation. Nat Commun. 2018;9(1):449.
151. Lee HP, Gu L, Mooney DJ, Levenston ME, Chaudhuri O. Mechanical confinement regulates cartilage matrix formation by chondrocytes. Nat Mater. 2017;16(12):1243-51.
152. Li W, Wu D, Hu D, Zhu S, Pan C, Jiao Y, et al. Stress-relaxing double-network hydrogel for chondrogenic differentiation of stem cells. Mater Sci Eng C Mater Biol Appl. 2020;107:110333.
153. Lee HJ, Seo Y, Kim HS, Lee JW, Lee KY. Regulation of the Viscoelastic Properties of Hyaluronate-Alginate Hybrid Hydrogel as an Injectable for Chondrocyte Delivery. ACS Omega. 2020;5(25):15567-75.
154. Lee K, Chen, Y., Li, X., Kawazoe, N., Yang, Y. and Chen, G. Influence of viscosity on chondrogenic differentiation of mesenchymal stem cells during 3D culture in viscous gelatin solution-embedded hydrogels. Journal of Materials Science & Technology. 2020.
155. McBride SH, Falls T, Knothe Tate ML. Modulation of stem cell shape and fate B: mechanical modulation of cell shape and gene expression. Tissue Eng Part A. 2008;14(9):1573-80.
156. Guilak F, Cohen DM, Estes BT, Gimble JM, Liedtke W, Chen CS. Control of stem cell fate by physical interactions with the extracellular matrix. Cell Stem Cell. 2009;5(1):17-26.
157. Tamaddon M, Burrows M, Ferreira SA, Dazzi F, Apperley JF, Bradshaw A, et al. Monomeric, porous type II collagen scaffolds promote chondrogenic differentiation of human bone marrow mesenchymal stem cells in vitro. Sci Rep. 2017;7:43519.
158. Matsiko A, Gleeson JP, O'Brien FJ. Scaffold mean pore size influences mesenchymal stem cell chondrogenic differentiation and matrix deposition. Tissue Eng Part A. 2015;21(3-4):486-97.
159. Nehrer S, Breinan HA, Ramappa A, Young G, Shortkroff S, Louie LK, et al. Matrix collagen type and pore size influence behaviour of seeded canine chondrocytes. Biomaterials. 1997;18(11):769-76.
160. Conrad B, Han LH, Yang F. Gelatin-Based Microribbon Hydrogels Accelerate Cartilage Formation by Mesenchymal Stem Cells in Three Dimensions. Tissue Eng Part A. 2018;24(21-22):1631-40.

161. Han L, Yu, S., Wang, T., Behn, A. and Yang, F. Tissue Engineering: Microribbon-Like Elastomers for Fabricating Macroporous and Highly Flexible Scaffolds that Support Cell Proliferation in 3D. Advanced Functional Materials. 2013;23(3):266.
162. Dalby MJ, Gadegaard N, Oreffo RO. Harnessing nanotopography and integrin-matrix interactions to influence stem cell fate. Nat Mater. 2014;13(6):558-69.
163. Oh S, Daraio C, Chen LH, Pisanic TR, Finones RR, Jin S. Significantly accelerated osteoblast cell growth on aligned TiO2 nanotubes. J Biomed Mater Res A. 2006;78(1):97-103.
164. Sjostrom T, Dalby MJ, Hart A, Tare R, Oreffo RO, Su B. Fabrication of pillar-like titania nanostructures on titanium and their interactions with human skeletal stem cells. Acta Biomater. 2009;5(5):1433-41.
165. Kim HN, Jiao A, Hwang NS, Kim MS, Kang DH, Kim DH, et al. Nanotopography-guided tissue engineering and regenerative medicine. Adv Drug Deliv Rev. 2013;65(4):536-58.
166. Cao B, Peng Y, Liu X, Ding J. Effects of Functional Groups of Materials on Nonspecific Adhesion and Chondrogenic Induction of Mesenchymal Stem Cells on Free and Micropatterned Surfaces. ACS Appl Mater Interfaces. 2017;9(28):23574-85.
167. Kim IL, Khetan S, Baker BM, Chen CS, Burdick JA. Fibrous hyaluronic acid hydrogels that direct MSC chondrogenesis through mechanical and adhesive cues. Biomaterials. 2013;34(22):5571-80.
168. Metavarayuth K, Maturavongsadit P, Chen X, Sitasuwan P, Lu L, Su J, et al. Nanotopographical Cues Mediate Osteogenesis of Stem Cells on Virus Substrates through BMP-2 Intermediate. Nano Lett. 2019;19(12):8372-80.
169. Li Z, Cao B, Wang X, Ye K, Li S, Ding J. Effects of RGD nanospacing on chondrogenic differentiation of mesenchymal stem cells. J Mater Chem B. 2015;3(26):5197-209.
170. Gegg C, Yang F. Spatially patterned microribbon-based hydrogels induce zonally-organized cartilage regeneration by stem cells in 3D. Acta Biomater. 2020;101:196-205.
171. Nguyen LH, Kudva AK, Saxena NS, Roy K. Engineering articular cartilage with spatially-varying matrix composition and mechanical properties from a single stem cell population using a multi-layered hydrogel. Biomaterials. 2011;32(29):6946-52.
172. Studle C, Vallmajo-Martin Q, Haumer A, Guerrero J, Centola M, Mehrkens A, et al. Spatially confined induction of endochondral ossification by functionalized hydrogels for ectopic engineering of osteochondral tissues. Biomaterials. 2018;171:219-29.
173. Park YB, Ha CW, Kim JA, Rhim JH, Park YG, Chung JY, et al. Effect of Transplanting Various Concentrations of a Composite of Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells and Hyaluronic Acid Hydrogel on Articular Cartilage Repair in a Rabbit Model. PLoS One. 2016;11(11):e0165446.
174. Leijten J, Teixeira LS, Bolander J, Ji W, Vanspauwen B, Lammertyn J, et al. Bioinspired seeding of biomaterials using three dimensional microtissues induces chondrogenic stem cell differentiation and cartilage formation under growth factor free conditions. Sci Rep. 2016;6:36011.
175. Liu J, Yu C, Chen Y, Cai H, Lin H, Sun Y, et al. Fast fabrication of stable cartilage-like tissue using collagen hydrogel microsphere culture. J Mater Chem B. 2017;5(46):9130-40.

176. Rogan H, Ilagan F, Yang F. Comparing Single Cell Versus Pellet Encapsulation of Mesenchymal Stem Cells in Three-Dimensional Hydrogels for Cartilage Regeneration. Tissue Eng Part A. 2019;25(19-20):1404-12.
177. Huang X, Hou Y, Zhong L, Huang D, Qian H, Karperien M, et al. Promoted Chondrogenesis of Cocultured Chondrocytes and Mesenchymal Stem Cells under Hypoxia Using In-situ Forming Degradable Hydrogel Scaffolds. Biomacromolecules. 2018;19(1):94-102.
178. Lai JH, Kajiyama G, Smith RL, Maloney W, Yang F. Stem cells catalyze cartilage formation by neonatal articular chondrocytes in 3D biomimetic hydrogels. Sci Rep. 2013;3:3553.
179. Amann E, Wolff P, Breel E, van Griensven M, Balmayor ER. Hyaluronic acid facilitates chondrogenesis and matrix deposition of human adipose derived mesenchymal stem cells and human chondrocytes co-cultures. Acta Biomater. 2017;52:130-44.
180. Cochis A, Grad S, Stoddart MJ, Fare S, Altomare L, Azzimonti B, et al. Bioreactor mechanically guided 3D mesenchymal stem cell chondrogenesis using a biocompatible novel thermo-reversible methylcellulose-based hydrogel. Sci Rep. 2017;7:45018.
181. Cao W, Lin W, Cai H, Chen Y, Man Y, Liang J, et al. Dynamic mechanical loading facilitated chondrogenic differentiation of rabbit BMSCs in collagen scaffolds. Regen Biomater. 2019;6(2):99-106.
182. Lin S, Lee WYW, Feng Q, Xu L, Wang B, Man GCW, et al. Synergistic effects on mesenchymal stem cell-based cartilage regeneration by chondrogenic preconditioning and mechanical stimulation. Stem Cell Res Ther. 2017;8(1):221.
183. Kowsari-Esfahan R, Jahanbakhsh A, Saidi MS, Bonakdar S. A microfabricated platform for the study of chondrogenesis under different compressive loads. J Mech Behav Biomed Mater. 2018;78:404-13.
184. Aisenbrey EA, Bryant SJ. Mechanical loading inhibits hypertrophy in chondrogenically differentiating hMSCs within a biomimetic hydrogel. J Mater Chem B. 2016;4(20):3562-74.
185. Aisenbrey EA, Bilousova G, Payne K, Bryant SJ. Dynamic mechanical loading and growth factors influence chondrogenesis of induced pluripotent mesenchymal progenitor cells in a cartilage-mimetic hydrogel. Biomater Sci. 2019;7(12):5388-403.
186. Aisenbrey EA, Bryant SJ. The role of chondroitin sulfate in regulating hypertrophy during MSC chondrogenesis in a cartilage mimetic hydrogel under dynamic loading. Biomaterials. 2019;190-191:51-62.
187. Sahu N, Budhiraja G, Subramanian A. Preconditioning of mesenchymal stromal cells with low-intensity ultrasound: influence on chondrogenesis and directed SOX9 signaling pathways. Stem Cell Res Ther. 2020;11(1):6.
188. Thoms BL, Dudek KA, Lafont JE, Murphy CL. Hypoxia promotes the production and inhibits the destruction of human articular cartilage. Arthritis Rheum. 2013;65(5):1302-12.
189. Robins JC, Akeno N, Mukherjee A, Dalal RR, Aronow BJ, Koopman P, et al. Hypoxia induces chondrocyte-specific gene expression in mesenchymal cells in association with transcriptional activation of Sox9. Bone. 2005;37(3):313-22.
190. Sathy BN, Daly A, Gonzalez-Fernandez T, Olvera D, Cunniffe G, McCarthy HO, et al. Hypoxia mimicking hydrogels to regulate the fate of transplanted stem cells. Acta Biomater. 2019;88:314-24.

191. Wang Y, Wu, S., Kuss, M., Streubel, P. and Duan, B. Effects of Hydroxyapatite and Hypoxia on Chondrogenesis and Hypertrophy in 3D Bioprinted ADMSC Laden Constructs. ACS Biomaterials Science & Engineering. 2017;3(5):826-35.
192. Motornov M, Roiter, Y., Tokarev, I. and Minko, S. Stimuli-responsive nanoparticles, nanogels and capsules for integrated multifunctional intelligent systems. Progress in Polymer Science. 2010;35(1-2):174-211.
193. Ji AR, Ku SY, Cho MS, Kim YY, Kim YJ, Oh SK, et al. Reactive oxygen species enhance differentiation of human embryonic stem cells into mesendodermal lineage. Exp Mol Med. 2010;42(3):175-86.
194. Lu Z, Liu S, Le Y, Qin Z, He M, Xu F, et al. An injectable collagen-genipin-carbon dot hydrogel combined with photodynamic therapy to enhance chondrogenesis. Biomaterials. 2019;218:119190.
195. Popa EG, Santo VE, Rodrigues MT, Gomes ME. Magnetically-Responsive Hydrogels for Modulation of Chondrogenic Commitment of Human Adipose-Derived Stem Cells. Polymers (Basel). 2016;8(2).
196. Huang J, Liang, Y., Huang, Z., Zhao, P., Liang, Q., Liu, Y., Duan, L., Liu, W., Zhu, F., Bian, L., Xia, J., Xiong, J. and Wang, D. Magnetic Enhancement of Chondrogenic Differentiation of Mesenchymal Stem Cells. ACS Biomaterials Science & Engineering. 2019;5(5):2200-7.
197. Green JD, Tollemar V, Dougherty M, Yan Z, Yin L, Ye J, et al. Multifaceted signaling regulators of chondrogenesis: Implications in cartilage regeneration and tissue engineering. Genes Dis. 2015;2(4):307-27.
198. Zhao Q, Eberspaecher H, Lefebvre V, De Crombrugghe B. Parallel expression of Sox9 and Col2a1 in cells undergoing chondrogenesis. Dev Dyn. 1997;209(4):377-86.
199. Akiyama H. Control of chondrogenesis by the transcription factor Sox9. Mod Rheumatol. 2008;18(3):213-9.
200. Akiyama H, Chaboissier MC, Martin JF, Schedl A, de Crombrugghe B. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev. 2002;16(21):2813-28.
201. Hino K, Saito A, Kido M, Kanemoto S, Asada R, Takai T, et al. Master regulator for chondrogenesis, Sox9, regulates transcriptional activation of the endoplasmic reticulum stress transducer BBF2H7/CREB3L2 in chondrocytes. J Biol Chem. 2014;289(20):13810-20.
202. Shi S, Wang C, Acton AJ, Eckert GJ, Trippel SB. Role of sox9 in growth factor regulation of articular chondrocytes. J Cell Biochem. 2015;116(7):1391-400.
203. Pan Q, Yu Y, Chen Q, Li C, Wu H, Wan Y, et al. Sox9, a key transcription factor of bone morphogenetic protein-2-induced chondrogenesis, is activated through BMP pathway and a CCAAT box in the proximal promoter. J Cell Physiol. 2008;217(1):228-41.
204. Liao J, Hu N, Zhou N, Lin L, Zhao C, Yi S, et al. Sox9 potentiates BMP2-induced chondrogenic differentiation and inhibits BMP2-induced osteogenic differentiation. PLoS One. 2014;9(2):e89025.
205. Furumatsu T, Tsuda M, Taniguchi N, Tajima Y, Asahara H. Smad3 induces chondrogenesis through the activation of SOX9 via CREB-binding protein/p300 recruitment. J Biol Chem. 2005;280(9):8343-50.
206. Akiyama H, Lyons JP, Mori-Akiyama Y, Yang X, Zhang R, Zhang Z, et al. Interactions between Sox9 and beta-catenin control chondrocyte differentiation. Genes Dev. 2004;18(9):1072-87.

207. Cheng A, Genever PG. SOX9 determines RUNX2 transactivity by directing intracellular degradation. J Bone Miner Res. 2010;25(12):2680-9.
208. Mori-Akiyama Y, Akiyama H, Rowitch DH, de Crombrugghe B. Sox9 is required for determination of the chondrogenic cell lineage in the cranial neural crest. Proc Natl Acad Sci U S A. 2003;100(16):9360-5.
209. Leung VY, Gao B, Leung KK, Melhado IG, Wynn SL, Au TY, et al. SOX9 governs differentiation stage-specific gene expression in growth plate chondrocytes via direct concomitant transactivation and repression. PLoS Genet. 2011;7(11):e1002356.
210. Kumar D, Lassar AB. The transcriptional activity of Sox9 in chondrocytes is regulated by RhoA signaling and actin polymerization. Mol Cell Biol. 2009;29(15):4262-73.
211. Tan Y, Xu Q, Li Y, Mao X, Zhang K. Crosstalk between the p38 and TGF-beta signaling pathways through TbetaRI, TbetaRII and Smad3 expression in plancental choriocarcinoma JEG-3 cells. Oncol Lett. 2014;8(3):1307-11.
212. Ebisawa K, Hata K, Okada K, Kimata K, Ueda M, Torii S, et al. Ultrasound enhances transforming growth factor beta-mediated chondrocyte differentiation of human mesenchymal stem cells. Tissue Eng. 2004;10(5-6):921-9.
213. Rozen-Zvi B, Hayashida T, Hubchak SC, Hanna C, Platanias LC, Schnaper HW. TGF-beta/Smad3 activates mammalian target of rapamycin complex-1 to promote collagen production by increasing HIF-1alpha expression. Am J Physiol Renal Physiol. 2013;305(4):F485-94.
214. Hanai J, Chen LF, Kanno T, Ohtani-Fujita N, Kim WY, Guo WH, et al. Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Calpha promoter. J Biol Chem. 1999;274(44):31577-82.
215. Alliston T, Choy L, Ducy P, Karsenty G, Derynck R. TGF-beta-induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. EMBO J. 2001;20(9):2254-72.
216. Wang J, Wang X, Holz JD, Rutkowski T, Wang Y, Zhu Z, et al. Runx1 is critical for PTH-induced onset of mesenchymal progenitor cell chondrogenic differentiation. PLoS One. 2013;8(9):e74255.
217. Wang Y, Belflower RM, Dong YF, Schwarz EM, O'Keefe RJ, Drissi H. Runx1/AML1/Cbfa2 mediates onset of mesenchymal cell differentiation toward chondrogenesis. J Bone Miner Res. 2005;20(9):1624-36.
218. Johnson K, Zhu S, Tremblay MS, Payette JN, Wang J, Bouchez LC, et al. A stem cell-based approach to cartilage repair. Science. 2012;336(6082):717-21.
219. Gorlin JB, Yamin R, Egan S, Stewart M, Stossel TP, Kwiatkowski DJ, et al. Human endothelial actin-binding protein (ABP-280, nonmuscle filamin): a molecular leaf spring. J Cell Biol. 1990;111(3):1089-105.
220. Connelly JT, Garcia AJ, Levenston ME. Interactions between integrin ligand density and cytoskeletal integrity regulate BMSC chondrogenesis. J Cell Physiol. 2008;217(1):145-54.
221. Goessling W, North TE, Loewer S, Lord AM, Lee S, Stoick-Cooper CL, et al. Genetic interaction of PGE2 and Wnt signaling regulates developmental specification of stem cells and regeneration. Cell. 2009;136(6):1136-47.
222. Kirton JP, Crofts NJ, George SJ, Brennan K, Canfield AE. Wnt/beta-catenin signaling stimulates chondrogenic and inhibits adipogenic differentiation of pericytes: potential relevance to vascular disease? Circ Res. 2007;101(6):581-9.

223. Ryu JH, Kim SJ, Kim SH, Oh CD, Hwang SG, Chun CH, et al. Regulation of the chondrocyte phenotype by beta-catenin. Development. 2002;129(23):5541-50.
224. Tufan AC, Tuan RS. Wnt regulation of limb mesenchymal chondrogenesis is accompanied by altered N-cadherin-related functions. FASEB J. 2001;15(8):1436-8.
225. Hay E, Laplantine E, Geoffroy V, Frain M, Kohler T, Muller R, et al. N-cadherin interacts with axin and LRP5 to negatively regulate Wnt/beta-catenin signaling, osteoblast function, and bone formation. Mol Cell Biol. 2009;29(4):953-64.
226. Liu Q, Wang W, Zhang L, Zhao L, Song W, Duan X, et al. Involvement of N-cadherin/beta-catenin interaction in the micro/nanotopography induced indirect mechanotransduction. Biomaterials. 2014;35(24):6206-18.
227. Tuli R, Tuli S, Nandi S, Huang X, Manner PA, Hozack WJ, et al. Transforming growth factor-beta-mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk. J Biol Chem. 2003;278(42):41227-36.
228. Ling L, Nurcombe V, Cool SM. Wnt signaling controls the fate of mesenchymal stem cells. Gene. 2009;433(1-2):1-7.
229. Baron R, Kneissel M. WNT signaling in bone homeostasis and disease: from human mutations to treatments. Nat Med. 2013;19(2):179-92.
230. Reinhold MI, Kapadia RM, Liao Z, Naski MC. The Wnt-inducible transcription factor Twist1 inhibits chondrogenesis. J Biol Chem. 2006;281(3):1381-8.
231. Fang M, Alfieri CM, Hulin A, Conway SJ, Yutzey KE. Loss of beta-catenin promotes chondrogenic differentiation of aortic valve interstitial cells. Arterioscler Thromb Vasc Biol. 2014;34(12):2601-8.
232. Kormish JD, Sinner D, Zorn AM. Interactions between SOX factors and Wnt/beta-catenin signaling in development and disease. Dev Dyn. 2010;239(1):56-68.
233. Topol L, Chen W, Song H, Day TF, Yang Y. Sox9 inhibits Wnt signaling by promoting beta-catenin phosphorylation in the nucleus. J Biol Chem. 2009;284(5):3323-33.
234. Faux MC, Coates JL, Kershaw NJ, Layton MJ, Burgess AW. Independent interactions of phosphorylated beta-catenin with E-cadherin at cell-cell contacts and APC at cell protrusions. PLoS One. 2010;5(11):e14127.
235. Wu SC, Chen CH, Wang JY, Lin YS, Chang JK, Ho ML. Hyaluronan size alters chondrogenesis of adipose-derived stem cells via the CD44/ERK/SOX-9 pathway. Acta Biomater. 2018;66:224-37.
236. Leung T, Chen XQ, Manser E, Lim L. The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. Mol Cell Biol. 1996;16(10):5313-27.
237. Allen JL, Cooke ME, Alliston T. ECM stiffness primes the TGFbeta pathway to promote chondrocyte differentiation. Mol Biol Cell. 2012;23(18):3731-42.
238. Hensel N, Baskal S, Walter LM, Brinkmann H, Gernert M, Claus P. ERK and ROCK functionally interact in a signaling network that is compensationally upregulated in Spinal Muscular Atrophy. Neurobiol Dis. 2017;108:352-61.
239. Yang C, Tibbitt MW, Basta L, Anseth KS. Mechanical memory and dosing influence stem cell fate. Nat Mater. 2014;13(6):645-52.
240. Karystinou A, Roelofs AJ, Neve A, Cantatore FP, Wackerhage H, De Bari C. Yes-associated protein (YAP) is a negative regulator of chondrogenesis in mesenchymal stem cells. Arthritis Res Ther. 2015;17:147.
241. Nie P, Li, Y., Suo, H., Jiang, N., Yu, D. and Fang, B.,. Dasatinib Promotes Chondrogenic Differentiation of Human Mesenchymal Stem Cells via the Src/Hippo-

YAP Signaling Pathway. ACS Biomaterials Science & Engineering. 2019;5(10):5255-65.