

1 Engineering High-Quality Cartilage Microtissues 2 using Hydrocortisone Functionalised Microwells

3 Ross Burdis^{1,2,3}, Gabriela Soares Kronemberger^{1,2,3}, and Daniel J. Kelly^{1,2,3,4} *

4 ¹ Trinity Centre for Biomedical Engineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Ireland.

5 ² Department of Mechanical, Manufacturing and Biomedical Engineering, School of Engineering, Trinity College
6 Dublin, Ireland.

7 ³ Advanced Materials and Bioengineering Research Centre (AMBER), Royal College of Surgeons in Ireland and
8 Trinity College Dublin, Ireland.

9 ⁴ Department of Anatomy and Regenerative Medicine, Royal College of Surgeons in Ireland, Dublin, Ireland

10 * Corresponding Author, **Email: kellyd9@tcd.ie**

11 **Abstract**

12 Engineering clinically-relevant musculoskeletal tissues at a human scale is a considerable challenge.
13 Developmentally-inspired scaffold-free approaches for engineering cartilage tissues have shown great
14 promise in recent years, enabling the generation of highly biomimetic tissues. Despite the relative
15 success of these approaches, the absence of a supporting scaffold or hydrogel creates challenges in
16 the development of large scale tissues. Combining numerous scaled-down tissue units (herein termed
17 *microtissues*) into a larger macrotissue represents a promising strategy to address this challenge. The
18 overall success of such approaches, however, relies on the development of strategies to support the
19 robust and consistent chondrogenic differentiation of clinically relevant cell sources such as
20 mesenchymal stem/stromal cells (MSCs) within microwell arrays to biofabricate numerous
21 microtissues rich in cartilage-specific extracellular matrix components. In this paper, we first describe
22 a simple method to manufacture cartilage microtissues at various scales using novel microwell array
23 stamps. This system allows the rapid and reliable generation of cartilage microtissues, and can be used
24 as a platform to study microtissue phenotype and development. Based on the unexpected discovery
25 that Endothelial Growth Medium (EGM) enhanced MSC aggregation and chondrogenic capacity within
26 the microwell arrays, this work also sought to identify soluble factors within the media capable of
27 supporting robust differentiation using heterogeneous MSC populations. Hydrocortisone was found
28 to be the key factor within EGM that enhanced the chondrogenic capacity of MSCs within these
29 microwell arrays. This strategy represents a promising means of generating large numbers of high-
30 quality, scaffold-free cartilage microtissues for diverse biofabrication applications.

31 **Keywords:** Mesenchymal Stem/Stromal Cell, Chondrogenesis, Hydrocortisone,
32 Microtissue, Microwell, Biofabrication, Cartilage Tissue Engineering

33 Introduction

34 Unlike traditional scaffold or hydrogel-based tissue engineering strategies, scaffold-free approaches
35 are inherently reliant on the cell's own capacity to generate the bulk of the tissue/construct through
36 the deposition of extracellular matrix (ECM). Although this follows a developmentally inspired
37 paradigm and facilitates the generation of biomimetic *in vitro* cartilage tissues [1–10], in the absence
38 of an interstitial 'bulking' scaffold or hydrogel material, creating tissues of scale can be challenging.
39 Scaling down scaffold-free tissue units can support more robust differentiation, alleviate diffusion
40 gradients, and ultimately improve matrix deposition [11–14]. Despite the numerous biological
41 benefits associated with scaled-down 3D scaffold-free strategies [1,15,16], microtissues do not yet
42 represent an idealised solution whereby robust ECM biosynthesis is guaranteed. A number of different
43 stem/progenitor cell sources, including articular chondrocytes [13,17], mesenchymal stem/stromal
44 cells (MSCs) [18–21], periosteal derived stem cells [12], and induced pluripotent stem cell (iPSC)
45 derived cells [22], have been used for the biofabrication of cartilage microtissues. Multiple methods
46 for forming multicellular spheroids/microtissues have been described [23], leveraging various non-
47 adherent polymers [21,24–27] and hydrogels [12–14,28–33] as substrate materials. At present,
48 cartilage microtissues are typically formed in a medium-to-high throughput manner using microwell
49 moulds, whereby stem/progenitor cells are collected in the bottom of a non-adherent well and
50 undergo cellular self-assembly/self-organisation to form a cellular spheroid. Under the appropriate
51 exogenous soluble cues, the cells within these aggregates can be differentiated and begin to deposit
52 a tissue-specific extracellular matrix (ECM), generating a microtissue. An ideal platform for generating
53 microtissues for biofabrication can be defined as a scalable process, capable of supporting the
54 development of standardised spheroids with defined shape, size and phenotype which can be used as
55 part of a subsequent biofabrication strategy, such as bioprinting [34]. Closely coupled with the
56 suitability of the method of forming microtissue building-blocks, is the quality of the microtissue
57 formed. The chosen platform for generating microtissues should support key processes such as
58 differentiation, phenotype commitment/maintenance, and the capacity for subsequent tissue fusion
59 [34]. Evaluation of microtissue quality (ECM composition, cellular phenotype, and functionality) can
60 be carried out using biochemical, histological, gene expression, and fusion assays.

61 Cellular heterogeneity, particularly with human MSCs, can result in poor chondrogenesis and
62 impact the richness of any cartilage ECM generated *in vitro* [35]. Such donor-to-donor variation is well
63 documented throughout the literature and has been shown to directly affect the *in vivo* performance
64 of engineered cartilages [36]. Therefore, engineering numerous microtissues, rich in cartilage-specific
65 ECM, in a practical and economical manner is challenging using MSC populations considering their
66 inherently variable chondrogenic capacity. The increased interest in the use of cellular spheroids,

67 microtissues, and/or organoids as biological building-blocks for engineering functional osteochondral
68 tissues/organs demands the development of strategies ideally suited to the biofabrication of large
69 numbers of homogeneous and phenotypically defined microtissues. Engineering large numbers of
70 such microtissues using clinically relevant cell sources requires the careful consideration of culture
71 conditions that regulate key outcomes such as microtissue phenotype (e.g. cell types and specific
72 growth factors), quality (e.g. mitigating diffusion gradients and nutrient limitations) and size (e.g. cell
73 numbers) [37]. In the context of cartilage and osteochondral tissue engineering, early research in this
74 area has focused on the use of undifferentiated MSC aggregates [38,39]. These immature aggregates
75 do not mimic the complex native ECM, which may explain why they fail to promote the regeneration
76 of hyaline cartilage when implanted into pre-clinical models for chondral/osteochondral defects
77 [40,41]. Therefore new approaches for engineering high quality cartilage microtissues at scale using
78 clinically relevant cell sources are required.

79 With this in mind, generating cartilage and osteochondral tissues of scale using
80 microtissue/aggregate engineering will require the high-throughput production of consistently high-
81 quality cartilage microtissues. In particular, methods for consistently engineering quality cartilage
82 microtissues from diverse donors with different chondrogenic capacity is required. Without well-
83 defined markers for identifying MSCs within primary isolations, many tissue engineers use
84 uncharacterised cohorts of cells for generating tissues, which in turn can reduce the reliability and
85 quality of the engineered cartilages. The identification of a simple method for improving the
86 chondrogenic capacity of uncharacterised MSC populations, isolated from bone marrow, could help
87 to limit the variability seen in cartilage tissue engineering. Specifically, in the context of cartilage
88 microtissue/aggregate engineering, the identification of protocols combatable with microwell
89 platforms typically used in the biofabrication of such microtissues are required. Ultimately, these high-
90 quality cartilage microtissues can be used as building blocks to more efficiently engineer cartilage and
91 osteochondral tissues of scale. In this paper we first describe the design of two microwell arrays that
92 can be used to directly pattern a hydrogel into an ideal platform for engineering cartilage microtissues.
93 We demonstrate the capacity to consistently form spherical cell aggregates within both the medium-
94 and high-throughput microwell systems, generating cartilage microtissues of different
95 maturities/phenotypes. Based on a serendipitous observation that Endothelial Growth Medium (EGM)
96 enhanced MSC aggregation and chondrogenic capacity in bone-marrow derived MSCs (BMSCs), this
97 study also sought to elucidate the driving factor(s) supporting such differentiation within EGM.
98 Ultimately, our aim was to improve upon current chondrogenic culture regimes and create a novel
99 platform for engineering high-quality, scaffold-free cartilage microtissues at scale.

100 **Materials & Methods**

101 **Media Formulations**

102 *Expansion Medium “XPAN”*. XPAN is composed of high glucose Dulbecco’s modified eagle’s medium
103 (hgDMEM) GlutaMAX supplemented with 10 % v/v FBS, 100 U/mL penicillin, 100 µg/mL streptomycin
104 (all Gibco, Biosciences, Dublin, Ireland) and 5 ng/mL FGF2 (Prospect Bio).

105 *Endothelial Growth Medium (EGM)*. EGM is composed of Endothelial Cell Basal Medium-2 (EBM)
106 (Lonza) supplemented with MV Microvascular Endothelial Cell Growth Medium-2 BulletKit™ (Lonza).
107 As the concentration of the supplements added to EBM are proprietary information, the
108 concentrations for each are given as a %v/v. Fetal bovine serum (FBS) was added at 5 %,
109 Hydrocortisone (Hydro) was added at 0.04 %, human FGF-2 (FGF) was added at 0.4 %, vascular
110 endothelial growth factor (VEGF), recombinant human long R3 insulin like growth factor 1 (IGF),
111 ascorbic acid (AA), and human epidermal growth factor (EGF) were all added at 0.1 %. Finally,
112 gentamicin sulfate-Amphotericin (GA-1000) was added at 0.1 %.

113 *Chondrogenic Differentiation Medium (CDM)*. hgDMEM GlutaMAX supplemented with 100 U/mL
114 penicillin, 100 µg/mL streptomycin (both Gibco), 100 µg/mL sodium pyruvate, 40 µg/mL L-proline, 50
115 µg/mL L-ascorbic acid-2-phosphate, 4.7 µg/mL linoleic acid, 1.5 mg/mL bovine serum albumin, 1 X
116 insulin–transferrin–selenium (ITS), 100 nM dexamethasone (all from Sigma), 2.5 µg/mL amphotericin
117 B and 10 ng/mL of human transforming growth factor-β3 (TGF-β) (Peprotech, UK).

118 *Hypertrophic Differentiation Medium (HYP)* was composed of hgDMEM GlutaMAX supplemented with
119 100 U/ml penicillin, 100 µg/mL streptomycin (both Gibco), 1 × ITS, 4.7 µg/ml linoleic acid, 50 nM
120 thyroxine, 100 nM dexamethasone, 250 µM ascorbic acid, 7 mM β-glycerophosphate and 2.5 µg/mL
121 amphotericin B (all from Sigma).

122 **Bone Marrow Mesenchymal Stem/Stromal Cell (BMSC) Isolation**

123 *Goat BMSC (gBMSC) isolation*. gBMSCs were harvested under sterile conditions from the sternum of
124 skeletally mature, female, Saanen goats. Briefly, excised bone marrow was dissected into small pieces
125 using a scalpel. The marrow pieces were then gently rotated for 5 min in XPAN to help liberate the
126 cellular components. The culture medium was then aspirated and passed through a 70 µm cell sieve
127 prior to counting and plating at a density of 57×10^3 cells/cm² and expanded under hypoxic conditions
128 (37 °C in a humidified atmosphere with 5 % CO₂ and 5 % O₂) for chondrogenic differentiation. Following
129 colony formation, gBMSCs were trypsinised using 0.25 % (w/v) Trypsin Ethylenediaminetetraacetic
130 acid (EDTA). gBMSCs for microtissues were expanded from an initial density of 5000 cells/cm² in XPAN
131 medium under physioxic conditions until P3.

132 *Human BMSC (hBMSC) isolation.* hBMSCs were isolated from unprocessed human bone marrow
133 (Lonza) on the basis of plastic adherence. Briefly, unprocessed bone marrow was plated at 2.5×10^5
134 cells/cm² (estimated approx. 4000 - 5000 MSCs/cm²) in XPAN medium and expanded under
135 physiological oxygen conditions (37 °C in a humidified atmosphere with 5 % CO₂ and 5 % O₂). Following
136 colony formation, hBMSCs were trypsinised using 0.25 % (w/v) Trypsin Ethylenediaminetetraacetic
137 acid (EDTA), hBMSCs were expanded in XPAN under physioxic conditions (5 % O₂) and aggregated into
138 pellets at P3.

139 **Microwell Platform Design, Fabrication and Validation**

140 Microwell stamps were designed using Solidworks CAD software. A summary of the dimensions of the
141 positive microwell stamps can be found in Figure 1A. Both the medium- and high-throughput
142 microwell arrays were designed to avoid flat sections between adjacent microwells. The medium-
143 throughput wells were designed to maintain discrete microtissues within individual microwells from
144 extended culture periods, and as such had a relatively deep well. In contrast, the microwells in the
145 high-throughput system were designed to maximise the number of microwells per macro-well, making
146 each well considerable smaller in dimension. The base of the high-throughput wells was designed to
147 be flat to maintain print fidelity as creating a curved or pointed base would require dimensions that
148 exceeded the printer's resolution (Both in x,y directions and laser spot size). Both microwell stamps
149 were fabricated using a Form 3 stereolithography (SLA) printer and the high-temperature resin (V2)
150 (both Formlabs, Massachusetts, United States). Prior to printing, a STL file for the part was prepared
151 using Preform 2.16.0 software (Formlabs, Massachusetts, United States), setting a 0.025 µm layer
152 height defined the resolution of the print. Completed parts were washed in propan-2-ol (Sigma
153 Aldrich) to clear any uncured resin, following which they were exposed to UV light (405 nm, 9.1 W)
154 (Form cure, Formlabs, Massachusetts, United States) for 120 min at 80°C to ensure complete
155 crosslinking. Before use, stamps were autoclave sterilised. Hydrogel microwells were moulded using
156 the same procedure as previously described [10]. Briefly, under sterile conditions, 4 % (w/v) molten
157 agarose was patterned using the microwell stamps within the wells of a 6 well-plate. Once cooled, the
158 stamps were removed and the agarose microwells soaked overnight in an appropriate media type
159 before cell seeding.

160 To validate the capacity of the hydrogel microwells to form spherical cellular aggregates and
161 cartilage microtissues, an appropriate cell suspensions (gBMSC) was pipetted into the macrowells to
162 achieve a final density of 4×10^3 cells/microwell. Well plates were then centrifuged at 700 x g for 5
163 minutes, and returned to physioxic conditions (37 °C in a humidified atmosphere with 5 % CO₂ and 5
164 % O₂) overnight to allow aggregation to occur (~18 hours). The following day, media was exchanged

165 to induce chondrogenesis (CDM) and changed every two days until the end point. For hypertrophic
166 cartilage microtissues, after 14 days of chondrogenic cultivation (CDM) HYP media was used for a
167 further 7 days to induce mineralisation of the cartilage microtissues.

168 **Experimental Design**

169 For all studies, microwells were seeded at a density that results in 4×10^3 cells/microtissue. Cell
170 expansion and cartilage microtissue cultivation took place at physiological oxygen conditions (37 °C in
171 a humidified atmosphere with 5 % CO₂ and 5 % O₂). With the exception of the first 24 hours, media
172 was exchanged every 2 days. Initial investigation using gBMSCs involved 21 Days of chondrogenic
173 culture. All studies using hMSCs were carried out over 7 days of chondrogenic cultivation.

174 *Control Group.* Here, cartilage microtissues are formed by seeding bMSCs into the microwells in XPAN.
175 The following day, the XPAN medium is carefully aspirated from the wells and replaced with CDM.

176 *EGM Group.* EGM was used to soak the agarose hydrogel microwells overnight prior to seeding. As
177 such, cells seeded into the microwells in the 'EGM' group were never directly exposed to EGM. The
178 seeding procedure and following chondrogenic culture was identical to the control group.

179 *Media Component Isolation Groups.* By means of determining the prominent factor within EGM that
180 aided in chondrogenesis, a screening study was undertaken. Each of the supplements listed in §2.1 for
181 EGM formulation were added at the correct concentration (%v/v) to both XPAN during pre-soaking
182 and seeding, as well as CDM during differentiation culture. Chondrogenic and experimental EGM
183 supplements were added to the basal media of CDM fresh prior to media exchange. Additionally,
184 blends of XPAN/CDM and EGM were used. In these groups EGM was supplemented as a 1× or 2×
185 formulation and then mixed 50/50 with either XPAN, for soaking and seeding, or with CDM (2×) for
186 chondrogenic differentiation. Summaries of the experimental groups and media compositions can be
187 found in supplementary figure 1 and supplementary table 1.

188 **Histological Analysis**

189 Samples were fixed using 4 % paraformaldehyde (PFA) solution overnight at 4 °C. After fixation,
190 samples were dehydrated in a graded series of ethanol solutions (70 % - 100 %), cleared in xylene, and
191 embedded in paraffin wax (all Sigma-Alrich). Prior to staining tissue sections (5 µm) were rehydrated.
192 Sections were stained with haematoxylin and eosin (H&E), 1 % (w/v) alcian blue 8GX in 0.1 M
193 hydrochloric acid (HCL) (AB) to visualise sulphated glycosaminoglycan (sGAG) content and counter-
194 stained with 0.1 % (w/v) nuclear fast red to determine cellular distribution, 0.1 % (w/v) picosirius red
195 (PSR) to visualise collagen deposition, and 1 % (w/v) alizarin red (pH 4.1) to determine mineral

196 deposition *via* calcium staining (all from Sigma-Aldrich). Stained sections were imaged using an Aperio
197 ScanScope slide scanner.

198 **Quantitative Biochemical Analysis**

199 Samples were washed in PBS after retrieval and the number of microtissues within each technical
200 replicate counted prior to digestion. A papain enzyme solution, 3.88 U/mL of papain enzyme in
201 100mM sodium phosphate buffer/5mM Na₂EDTA/10mM Lcysteine, pH 6.5 (all from Sigma–Aldrich),
202 was used to digest the samples at 60 °C for 18 hours. DNA content was quantified immediately after
203 digestion using Quant-iT™ PicoGreen® dsDNA Reagent and Kit (Molecular Probes, Biosciences). The
204 amount of sGAG was determined using the dimethylmethylene blue dye-binding assay (Blyscan,
205 Biocolor Ltd., Northern Ireland), with a chondroitin sulphate standard read using the Synergy HT multi-
206 detection micro-plate reader (BioTek Instruments, Inc) with a wavelength set to 656 nm. Total collagen
207 content was determined using a chloramine-T assay [42] to measure the hydroxyproline content and
208 calculated collagen content using a hydroxyproline-to-collagen ratio of 1:7.69. Briefly, samples were
209 mixed with 38 % HCL (Sigma) and incubated at 110 °C for 18 hours to allow hydrolysis to occur.
210 Samples were subsequently dried in a fume hood and the sediment reconstituted in ultra-pure H₂O.
211 2.82 % (w/v) Chloramine T and 0.05 % (w/v) 4-(Dimethylamino) benzaldehyde (both Sigma) were
212 added and the hydroxyproline content quantified with a trans-4-Hydroxy-L-proline (Fluka analytical)
213 standard using a Synergy HT multi-detection micro-plate reader at a wavelength of 570 nm (BioTek
214 Instruments, Inc).

215 **Image Quantification & Statistical Analysis**

216 Diameter measurement of growing microtissues were taken from microscope images (4×) using
217 ImageJ software. Statistical analysis was performed using GraphPad Prism software (GraphPad
218 Software, CA, USA). Analysis of differences between two groups at one timepoint was done using a
219 standard two-tailed t-test. For two groups over multiple time-points a one-way analysis of variance
220 (ANOVA) was performed. Numerical and graphical results are presented as mean ± standard deviation
221 unless stated otherwise. Significance was determined when $p < 0.05$.

222 Results

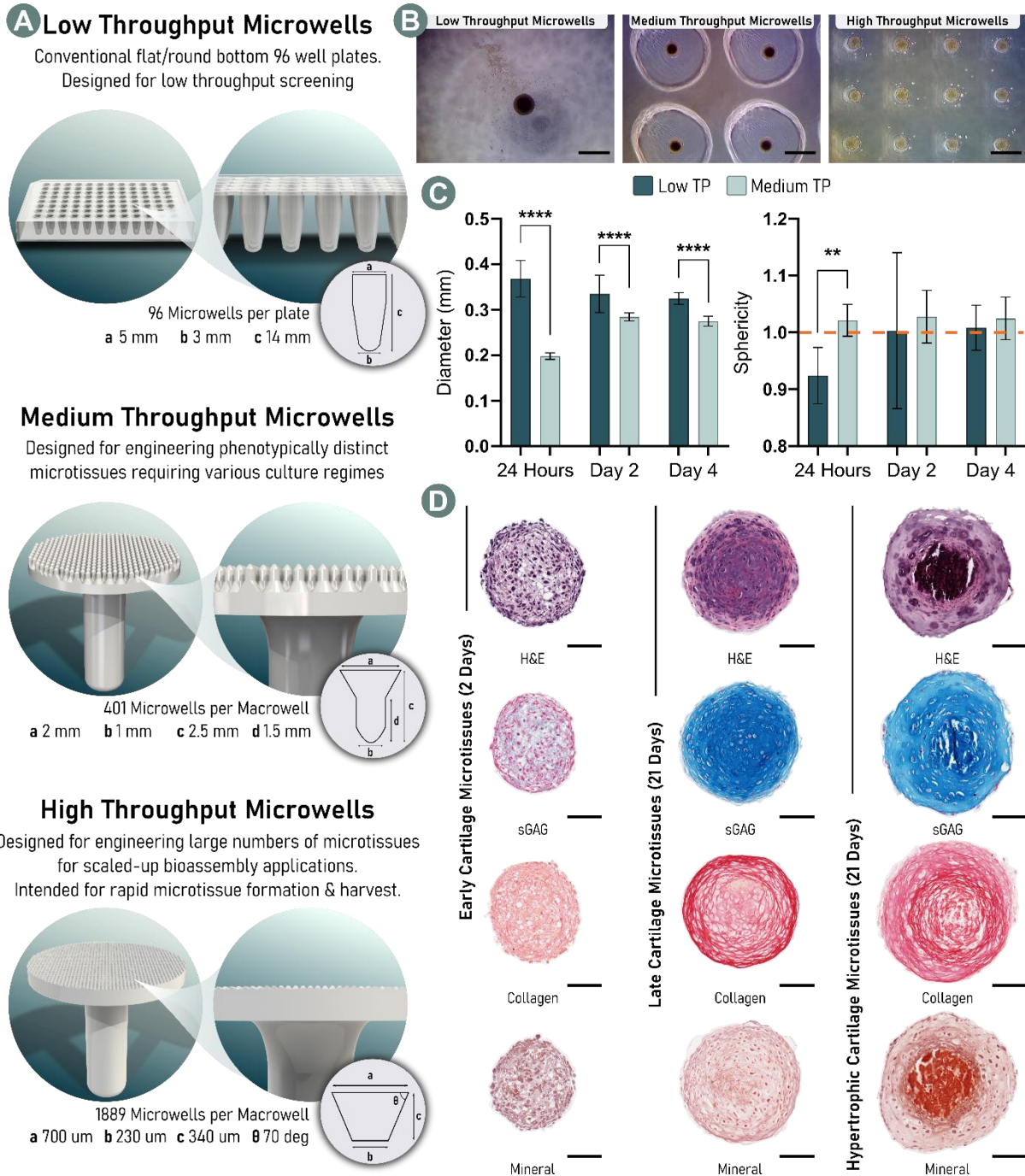


Figure 1. Microwell platform design and validation. A) Schematic representation of three platforms for generating spherical cartilage microtissues. Our medium- and high-throughput systems have been designed to directly pattern a hydrogel substrate within a conventional 6 well-plate. B) Microscopic images of spherical cellular aggregates formed after 2 days within each platform (Scale Bar = 500 μ m). C) Quantification of microtissue diameter and sphericity (orange line indicates a perfect sphere) after 24 hours, 2 days, and 4 days of cultivation within the low- and medium-throughput systems. Significant differences were tested using a Šidák's multiple comparisons test, ordinary two-way ANOVA, where; ** denotes $p < 0.01$ and **** denotes $p < 0.0001$, ($N = 10$, Mean \pm SD). D) Histological analysis of phenotypically distinct cartilage microtissues displaying markers of early-, late-, and hypertrophic-cartilage (Scale Bar = 100 μ m).

223 **Microwell Platforms for Microtissue Biofabrication**

224 We developed two microwell platforms ideally suited to engineer numerous cartilage microtissues
225 and compared these to standard 96 well plates (Figure 1A). Both the medium- and high-throughput
226 microwell stamps were designed to directly pattern an agarose hydrogel within a conventional 6 well-
227 plate [10]. The medium-throughput stamp generates 401 round bottom microwells with a sufficient
228 well depth to maintain discrete microtissues within individual microwells for extended culture periods
229 allowing for numerous media changes. Such platforms can be used for the engineering of cartilage
230 microtissues with various phenotypes (Figure 1D) and/or investigating microtissue development
231 under various culture conditions. Although all platforms support the formation of spherical cell
232 aggregates (Figure 1B), we demonstrated that our custom microwell platforms result in a more rapid
233 and reliable spheroid formation when compared to a conventional 96 well plate (Figure 1C). The
234 medium-throughput hydrogel microwell was hereon in used as the platform for investigating novel
235 culture conditions for supporting enhanced chondrogenesis.

236 **Endothelial Growth Media (EGM) Treatment Enhances Aggregation and Chondrogenesis of** 237 **BMSCs**

238 Soaking agarose hydrogel microwells with EGM prior to cell seeding appeared to have a rapid and
239 potent effect on the self-organisation of gBMSCs into a cellular spheroid. By day 2, gBMSC aggregates
240 were significantly larger, and microscopically appeared to include all of the cells which had been
241 seeded into the individual microwells (Figure 2Ai). In contrast, gBMSC aggregates generated within
242 XPAN soaked microwells had a smaller average diameter, with a large numbers of cells not coalescing
243 within the spheroid, instead appearing at the bottom of the microwell. These significant differences
244 in size were maintained throughout the culture period, resulting in a final average microtissue
245 diameter of $0.403 \pm 0.03 \mu\text{m}$ and $0.311 \pm 0.026 \mu\text{m}$ for EGM and XPAN soaked microwells respectively
246 (Figure 2Aii). Histologically, both microtissue cohorts exhibited canonical markers for chondrogenic
247 differentiation, with positive matrix staining for sGAG and collagen deposition. In the EGM pre-soak
248 group, the intensity of the staining indicated a richer cartilaginous ECM. Neither group stained positive
249 for calcium deposition, providing evidence that the cartilage has not yet progressed towards a mature
250 hypertrophic phenotype (Figure 2B). Biochemical evaluation demonstrated that there were
251 significantly higher levels of DNA, sGAG, and collagen per microtissue when the microwells were
252 soaked with EGM compared to XPAN. Additionally, the levels of sGAG and collagen deposited,
253 normalised to DNA content, demonstrated that EGM treatment resulted in a higher biosynthetic
254 output at a cellular level (Figure 2C). Collectively, these results indicated that EGM treatment resulted
255 in the generation of larger, more cellular microtissues containing higher levels of cartilage-specific

256 ECM components. Moreover, the cells within the microtissue demonstrated a higher synthetic output
 257 compared to those undergoing a traditional chondrogenic culture regime.

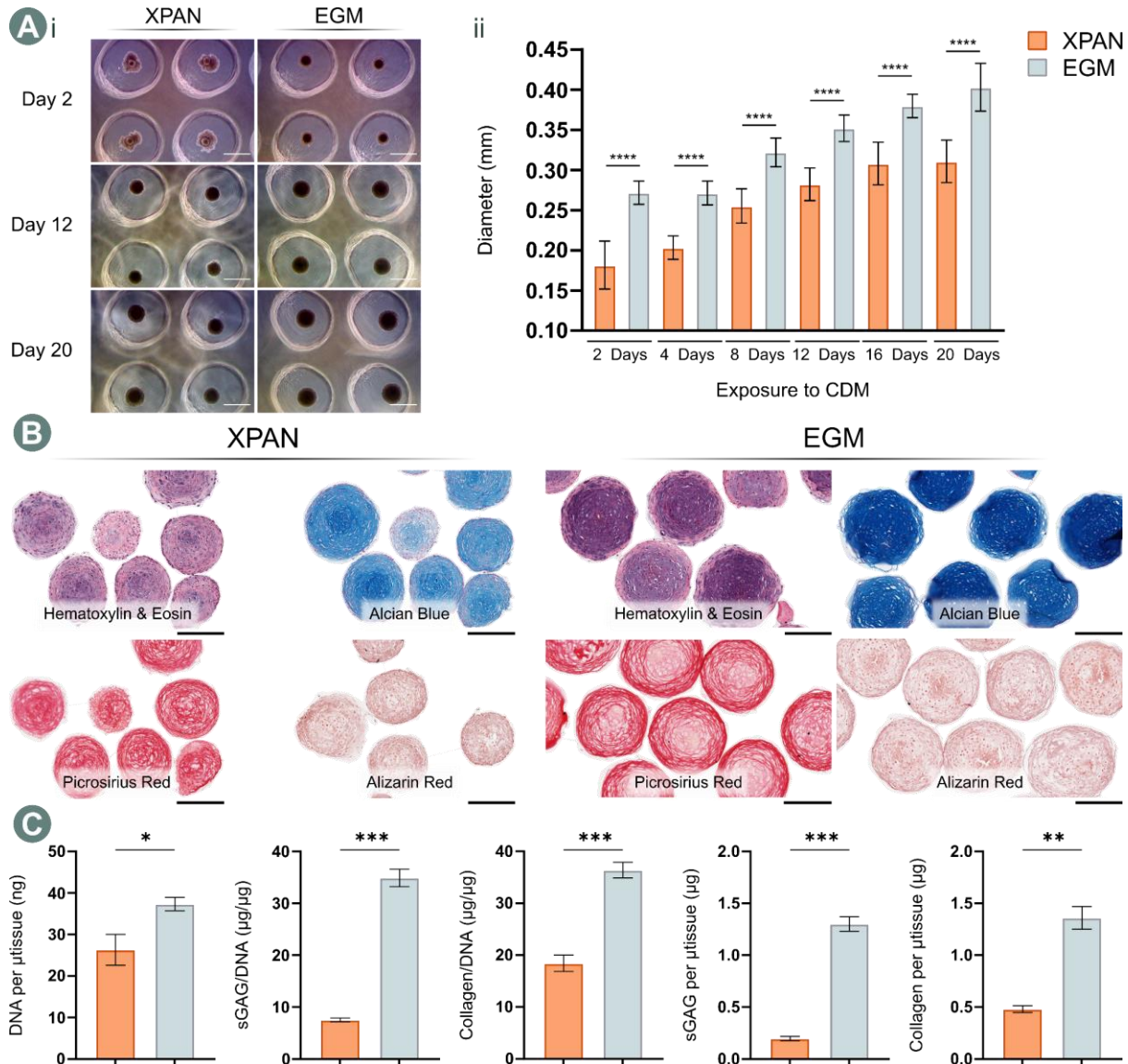


Figure 2. Soaking hydrogel microwells with EGM results in a richer matrix within cartilage microtissues. A) Microscopic images at days 2, 12, and 20 during chondrogenic culture (Scale bar = 500 μm), and quantification of diameter (ii) as a non-destructive metric for microtissue development. **** denotes significance when tested using a Šidák's multiple comparisons test, two-way ANOVA, where $p < 0.0001$, ($N = 20$, Mean \pm SD). B) Histological evaluation of cartilage microtissues after 21 days of chondrogenic culture (Scale bar = 200 μm). C) Biochemical quantification of the cartilage microtissues after 21 days. * denotes significance using a two-tailed, unpaired Welch's t-test, where; * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$ ($N = 3$, Mean \pm SD).

258 **Hydrocortisone Supports Enhanced Chondrogenesis in Human BMSCs**

259 EGM contains multiple factors that potentially enhance chondrogenesis of BMSCs in this microtissue
260 model (Supplementary table 1). This motivated an investigation to identify the predominant driving
261 factors within the EGM. Additionally, to improve its clinical relevance, this empirical study was
262 undertaken using hBMSCs. To this end, hBMSCs within the microwell system were cultured in media
263 supplemented with each factor used within the EGM formulation. These factors were added to both
264 the XPAN used during soak loading and seeding, as well as to CDM during chondrogenic induction. A
265 typical chondrogenic culture regime 'control' was also carried out, as well as EGM soak loading (here
266 termed 'EGM'), which was identical to the protocol shown to be effective in animal derived BMSCs.
267 After 7 days of *in vitro* chondrogenesis, differences in microtissue size were apparent microscopically
268 (Figure 3B). Cellular arrangement also appeared to vary within the microtissues depending on which
269 additional EGM supplement was provided (Figure 3A). Histologically, archetypal cartilage spheroids
270 were seen in the control, EGM, and hydrocortisone (Hydro) groups. Although other supplements did
271 not entirely suppress chondrogenesis, with sGAG and collagen deposition detected in all groups at
272 varying levels, they did result in condensed, highly cellular and atypical cartilage spheroids. When
273 compared to a standard chondrogenic culture regime (control), supplementation with hydrocortisone
274 resulted in significantly higher levels of DNA per microtissue, as well as a higher deposition of sGAG
275 per cell (Figure 3C). Although EGM soaking did not significantly influence the DNA levels within the
276 microtissues in hBMSCs, its effect on biosynthetic output did mirror observations made previously
277 with gBMSCs, whereby pre-soaking with EGM resulted in a significantly richer cartilaginous ECM
278 profile compared to standard chondrogenic culture conditions (control).

279 To confirm the effect of EGM and hydrocortisone treatment on hMSC chondrogenesis in this
280 microtissue system, the key groups from the above experiment were repeated using cells isolated
281 from a different human donor. Diameter measurements taken during the 7 days *in vitro* revealed
282 similar responses in terms of microtissue growth in both experimental groups (Figure 4A). Although
283 microtissues within these groups remained significantly larger than those under conventional
284 chondrogenic conditions, unlike in the previous study, the diameter of microtissues in the control
285 group also increased over the 7 days. Histologically, all groups supported robust chondrogenic
286 differentiation and the deposition of cartilage specific ECM components (Figure 4B). Biochemical
287 quantification of the cartilage microtissues indicated that significantly higher levels of sGAG/DNA and
288 sGAG/microtissue could be achieved using the EGM soak loading and hydrocortisone treatments
289 respectively. Despite this, there was no significant benefit in terms of collagen deposition for either
290 experimental group. Interestingly, the baseline chondrogenic capacity of the donor investigated
291 within this study appeared far superior to that of the previous hBMSCs. Under standard chondrogenic

292 conditions (control), the sGAG/DNA was 5.50 ± 0.175 , compared to 2.81 ± 0.236 for the previous
293 donor. The difference in collagen deposition per cell was more pronounced, with 25.9 ± 1.13
294 collagen/DNA for this donor versus 5.83 ± 0.887 collagen/DNA for the previous donor. This suggests
295 that the beneficial effects of such treatments are more pronounced when the baseline levels of
296 chondrogenesis are relatively low.

297

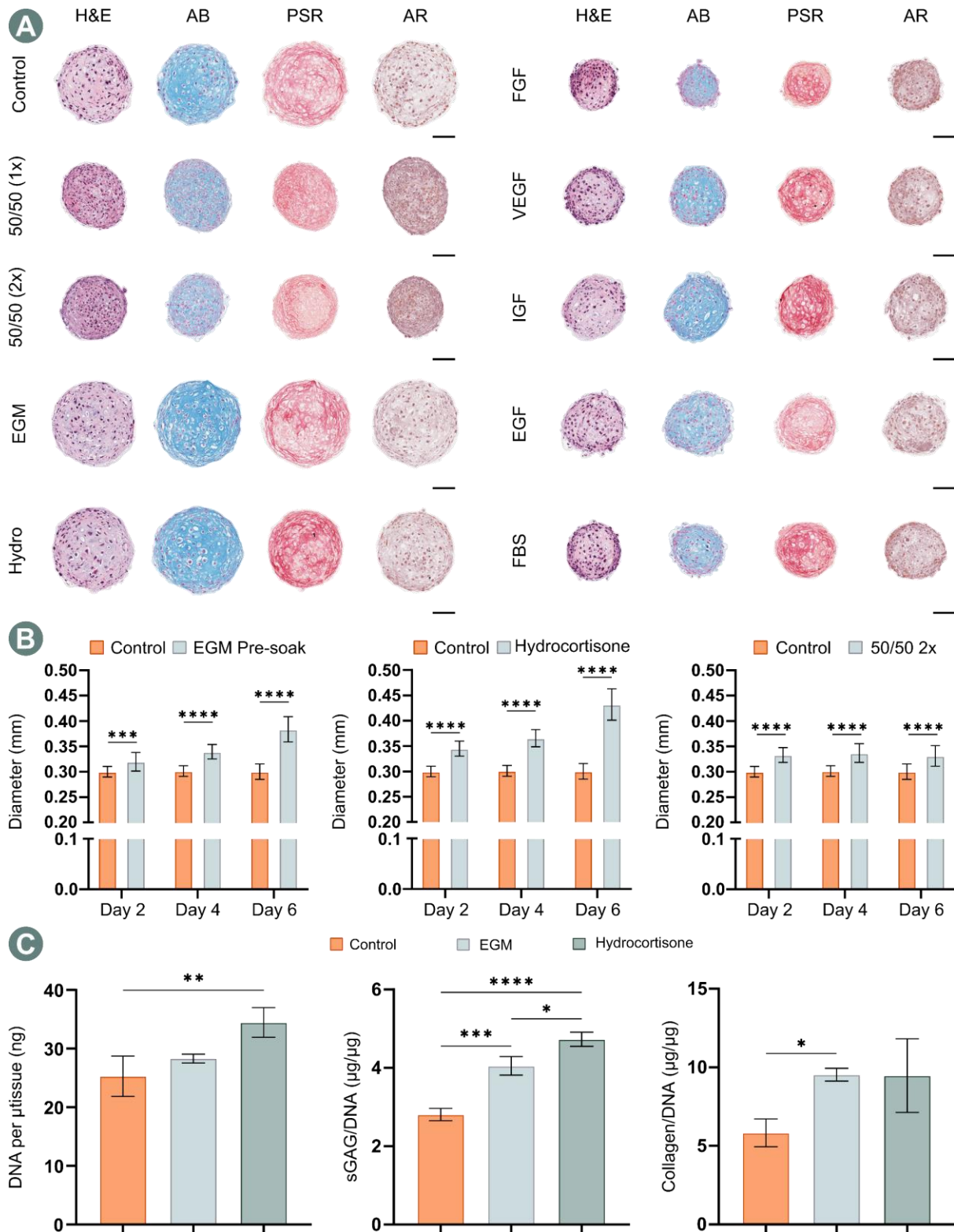


Figure 3. Hydrocortisone is the driving factor in EGM that improved chondrogenesis. A) Histological panel of microtissues, representing each EGM supplement, after 7 days of chondrogenic... (figure caption continued on following page)

culture (Scale Bar = 100 μ m). B) Diameter measurements during culture, * denotes significance when tested using a Šídák's multiple comparisons test, two-way ANOVA, where; *** indicates $p < 0.001$ and **** indicates $p < 0.0001$, ($N = 20$, Mean \pm SD). C) Biochemical quantification of the cartilage microtissues after 7 days of chondrogenic culture. Hydrocortisone treatment compared to typical chondrogenic conditions (control) and positive control group (EGM) demonstrated a significant increase in DNA content and sGAG deposition. * denotes significance using an Ordinary One-way ANOVA with a Tukey's multiple comparisons test, where; * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$, and **** indicates $p < 0.0001$ ($N = 3$, Mean \pm SD).

298

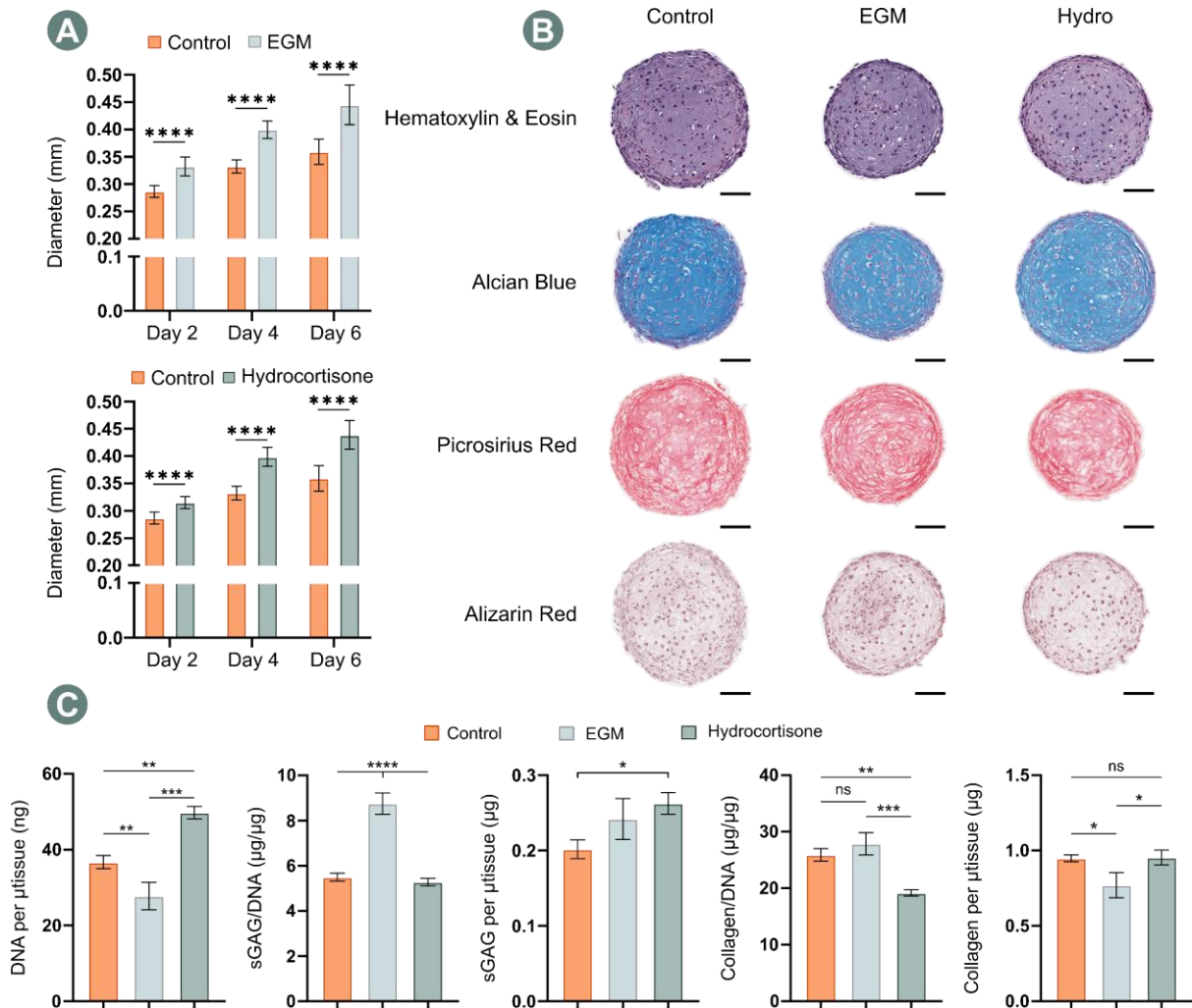


Figure 4. The effect of both EGM pre-soaking and hydrocortisone supplementation is lessened in a more chondrogenic hBMSC population. A) Quantification of microtissue diameter. **** denotes significance when tested using a Šídák's multiple comparisons test, two-way ANOVA, where $p < 0.0001$, ($N = 25$, Mean \pm SD). B) Histological evaluation of cartilage microtissues after 7 days of chondrogenic culture (Scale bar = 100 μ m). C) Biochemical quantification of the cartilage microtissues after 21 days. * denotes significance using an Ordinary One-way ANOVA with a Tukey's multiple comparisons test, where; ns indicates $p > 0.05$, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, and **** indicates $p < 0.0001$ ($N = 3$ for control & Hydro, $N = 4$ for EGM, Mean \pm SD).

299 Discussion

300 This study aimed to establish a novel protocol for ensuring the generation of high-quality cartilage
301 microtissues. Soaking hydrogel microwells in fully supplemented EGM a day prior to seeding gBMSCs
302 was found to enhance cellular aggregation and cause a significant improvement in chondrogenesis. At
303 day 2, all cells within the microwells of the EGM group had coalesced, forming large spherical
304 aggregates. In contrast, a standard chondrogenic protocol yielded relatively small cell aggregates with
305 a large number of unengaged cells surrounding the spheroids. By day 21 of culture, histological and
306 biochemical evaluation indicated that a more cartilaginous ECM could be generated by soak loading
307 the microwells with EGM prior to seeding. Cartilage matrix components (sGAG and collagen) were
308 more extensively deposited in the EGM group compared to the control condition. Moreover, the
309 increased abundance of ECM proteins was not only due to more cellular microtissues, as evident by
310 higher DNA content after 21 days of culture, but also as a result of the increased biosynthetic output
311 of the resident cell population within the EGM microtissues. Collectively, this preliminary study
312 indicated that factor(s) within EGM provide potent cues capable of improving the chondrogenic
313 capacity of an uncharacterised BMSCs population.

314 Next, we sought to determine if a single component within the EGM was primarily responsible
315 for the aforementioned results using more clinically relevant hBMSCs. Within the supplement profile
316 of EGM, basic FGF/FGF-2 (FGF) and insulin-like growth factor (IGF) were potential candidates for
317 driving improved chondrogenesis. FGF is known to maintain MSCs in an immature state, enhance their
318 proliferation during *in vitro* expansion and their subsequent differentiation potential [43].
319 Additionally, the treatment of hMSCs with FGF during expansion has given rise to enhanced
320 chondrogenesis [44,45]. Specifically, chondrogenic aggregates formed using cells treated with FGF
321 during monolayer expansion were larger and expressed higher proteoglycan content. Additionally,
322 FGF-treated cells have been formed into cartilage spheroids that lacked collagen type I and expressed
323 collagen type II in their periphery [45]. FGF signalling, although not critical for chondrogenesis, has
324 been associated with improved chondrogenic differentiation of hMSCs [46]. However, prolonged
325 treatment with FGF during MSC condensation and early chondrogenic differentiation has been shown
326 to inhibit chondrogenesis, whereas the addition of other isoforms, such as FGF-9, to chondrogenic
327 media has been shown to marginally increase matrix production during early chondrogenesis [47]. In
328 this study, all MSCs were exposed to FGF during expansion, however exposure to FGF during the first
329 7 days of chondrogenic differentiation did not enhance chondrogenesis and ECM production
330 (Supplementary Figure 2). IGF, when combined with TGF- β , is commonly discussed as a promoter of
331 chondrogenesis in MSCs [43,48]. IGF alone has been suggested to have similar chondrogenic effects
332 as TGF- β , stimulating proliferation, regulating apoptosis, and inducing the expression of chondrogenic

333 markers in BMSCs. Moreover, the two growth-factors have demonstrated additive effects, resulting
334 in gene expression analogous to human primary culture chondrocytes [49]. We failed to see a similar
335 results in this study, as there was no discernible benefit associated with supplementing CDM with IGF
336 (Supplementary Figure 2).

337 The growth factor hydrocortisone emerged as the principle driving factor within the EGM
338 supplement capable of promoting more robust chondrogenesis. Intra-articular injection of
339 glucocorticoids, such as hydrocortisone, is a longstanding means of managing arthritis. Primarily,
340 glucocorticoid therapy aims to provide symptomatic relief, reducing inflammation and pain within an
341 affected joint. However, the use of such steroidal agents has been discouraged for the treatment of
342 OA due to their undesirable effects on cartilage metabolism [50]. Despite this, chondroprotective
343 properties and other putative benefits of glucocorticoid treatment have been suggested. More
344 recently, the chondroprotective capacity of hydrocortisone has been found to be heavily dose-
345 dependent, with beneficial changes associated with low doses both *in vitro* and *in vivo*, whereas higher
346 doses result in deleterious effects [51]. *In vitro*, the exposure of MSCs to synthetic glucocorticoids for
347 the initiation of chondrogenesis has been well established through the use of dexamethasone (DEX)
348 [52,53]. The role of DEX in promoting chondrogenesis has been elucidated through studies
349 demonstrating that glucocorticoids directly regulate the expression of cartilage ECM genes and/or
350 enhance TGF- β -mediated effects on their expression. Specifically, a positive interaction between TGF-
351 β and glucocorticoid signalling pathways, which are mediated by the glucocorticoid receptor, have
352 been demonstrated during chondrogenesis [54]. The impact of hydrocortisone, an adrenocortical
353 hormone, is much less documented. It has been reported to be found in FBS, where it is important in
354 the modulation of MSC functions such as growth and adhesion [55]. As such, it is often included in
355 serum-free medium formulations. Additionally, hydrocortisone has been used to ‘activate’
356 multipotent MSCs for adipogenic differentiation, while its addition during passaging helps to preserve
357 the self-maintenance capacity of MSCs [56]. In the context of chondrogenesis, hydrocortisone
358 supplementation in 3D culture with human chondrocytes has been shown to optimise ECM
359 metabolism. In particular, exposure to physiological levels of hydrocortisone was linked with an
360 enhanced capacity to synthesise ECM components (aggrecan, collagen type II, and fibronectin) whilst
361 decreasing the activity of catabolic pathways (suppression of the IL1 catabolic pathway - reduced
362 intracellular IL1- α and - β as well as IL1RI) [57]. Collectively, these results indicate that corticosteroids
363 can be beneficially leveraged in the engineering of high-quality cartilage microtissues using MSCs.

364 Whilst the mechanism of action remains unclear, we present evidence that suggests
365 EGM/hydrocortisone treatment can improve the chondrogenic potential of heterogeneous MSC

366 populations. Investigation into the full effects of this novel chondrogenic protocol in terms of its
367 regulation/re-activation of MSC subpopulations, and/or its effect on chondrogenic genes that are co-
368 regulated *via* glucocorticoid receptors would provide interesting additional insight and could help
369 design enhanced chondrogenic cultures in the future. Given the evidence that selection of superior
370 chondrogenic donors *in vitro* can translate into improved *in vivo* outcomes [58,59], the data presented
371 in this work represents a simple alternative method for maximising the chondrogenic capacity of MSC
372 populations that exhibit inherently limited chondrogenesis. As such, effectively implementing this
373 novel protocol can result in the formation of high-quality cartilage microtissues. To this end, our
374 preliminary data (Supplementary Figure 3) suggests that exposure to hydrocortisone at a
375 concentration of 0.2 µg/mL may be beneficial for chondrogenic culture. This evidence, coupled with
376 our findings relating to the potency of EGM soak loading indicates that a similar soaking, or short-term
377 exposure strategies (<7 days) may be the most effective means of implementing hydrocortisone
378 treatment within a chondrogenic culture regime. Ultimately, this work provides a platform to generate
379 larger engineered cartilage through self-organisation of these high-quality building blocks without the
380 need for additional cells, unfeasible numbers of microtissue units, or compromising the quality of the
381 final construct.

382 **Conclusion**

383 Collectively, the results of this study indicate that pre-treatment *via* EGM pre-soaking or the
384 supplementation of chondrogenic differentiation medium with hydrocortisone can provide a simple
385 and potent means of improving chondrogenesis in heterogeneous MSC cohorts. This work could
386 enable the generation of more scalable engineered cartilages by ensuring the formation of high-
387 quality cartilage microtissue building blocks without the need for extensive cell immunophenotyping.

388 **Acknowledgements**

389 This publication was developed with the financial support of Science Foundation Ireland (SFI) under
390 grant number 12/RC/2278 and 17/SP/4721. This research is co-funded by the European Regional
391 Development Fund and SFI under Ireland's European Structural and Investment Fund. This research
392 has been co-funded by Johnson & Johnson 3D Printing Innovation & Customer Solutions, Johnson &
393 Johnson Services Inc.

394 References

- 395 [1] K.A. Athanasiou, R. Eswaramoorthy, P. Hadidi, J.C. Hu, Self-Organization and the Self-
396 Assembling Process in Tissue Engineering, *Annu. Rev. Biomed. Eng.* 15 (2013) 115–136.
397 <https://doi.org/10.1146/annurev-bioeng-071812-152423>.
- 398 [2] J.C. Hu, K.A. Athanasiou, A Self-Assembling Process in Articular Cartilage Tissue Engineering,
399 *Tissue Eng.* 12 (2006) 969–979. <https://doi.org/10.1089/ten.2006.12.969>.
- 400 [3] A.M. DeLise, L. Fischer, R.S. Tuan, Cellular interactions and signaling in cartilage development,
401 *Osteoarthr. Cartil.* 8 (2000) 309–334. <https://doi.org/10.1053/joca.1999.0306>.
- 402 [4] M. Centola, B. Tonnarelli, S. Schären, N. Glaser, A. Barbero, I. Martin, Priming 3D Cultures of
403 Human Mesenchymal Stromal Cells Toward Cartilage Formation Via Developmental
404 Pathways, *Stem Cells Dev.* 22 (2013) 2849–2858. <https://doi.org/10.1089/scd.2013.0216>.
- 405 [5] G. Ofek, C.M. Revell, J.C. Hu, D.D. Allison, K.J. Grande-Allen, K.A. Athanasiou, Matrix
406 development in self-assembly of articular cartilage, *PLoS One.* 3 (2008).
407 <https://doi.org/10.1371/journal.pone.0002795>.
- 408 [6] D.J. Responde, B. Arzi, R.M. Natoli, J.C. Hu, K.A. Athanasiou, Mechanisms underlying the
409 synergistic enhancement of self-assembled neocartilage treated with chondroitinase-ABC and
410 TGF- β 1, *Biomaterials.* 33 (2012) 3187–3194.
411 <https://doi.org/10.1016/j.biomaterials.2012.01.028>.
- 412 [7] J.J. Ng, Y. Wei, B. Zhou, J. Bernhard, S. Robinson, A. Burapachaisri, X.E. Guo, G. Vunjak-
413 Novakovic, Recapitulation of physiological spatiotemporal signals promotes in vitro formation
414 of phenotypically stable human articular cartilage, *Proc. Natl. Acad. Sci.* 114 (2017) 2556–
415 2561. <https://doi.org/10.1073/pnas.1611771114>.
- 416 [8] W.D. Lee, M.B. Hurtig, R.A. Kandel, W.L. Stanford, Membrane Culture of Bone Marrow
417 Stromal Cells Yields Better Tissue Than Pellet Culture for Engineering Cartilage-Bone
418 Substitute Biphasic Constructs in a Two-Step Process, *Tissue Eng. Part C Methods.* 17 (2011)
419 939–948. <https://doi.org/10.1089/ten.tec.2011.0147>.
- 420 [9] L. Zhang, P. Su, C. Xu, J. Yang, W. Yu, D. Huang, Chondrogenic differentiation of human
421 mesenchymal stem cells: A comparison between micromass and pellet culture systems,
422 *Biotechnol. Lett.* 32 (2010) 1339–1346. <https://doi.org/10.1007/s10529-010-0293-x>.
- 423 [10] J. Nulty, R. Burdis, D.J. Kelly, Biofabrication of Prevascularised Hypertrophic Cartilage
424 Microtissues for Bone Tissue Engineering, *Front. Bioeng. Biotechnol.* 9 (2021) 1–16.
425 <https://doi.org/10.3389/fbioe.2021.661989>.
- 426 [11] M. Bhattacharjee, J. Coburn, M. Centola, S. Murab, A. Barbero, D.L. Kaplan, I. Martin, S.
427 Ghosh, Tissue engineering strategies to study cartilage development, degeneration and
428 regeneration, *Adv. Drug Deliv. Rev.* 84 (2015) 107–122.
429 <https://doi.org/10.1016/j.addr.2014.08.010>.
- 430 [12] G. Nilsson Hall, L.F. Mendes, C. Gklava, L. Geris, F.P. Luyten, I. Papantoniou, Developmentally
431 Engineered Callus Organoid Bioassemblies Exhibit Predictive In Vivo Long Bone Healing, *Adv.*
432 *Sci.* 7 (2020) 1–16. <https://doi.org/10.1002/advs.201902295>.
- 433 [13] L. De Moor, E. Beyls, H. Declercq, Scaffold Free Microtissue Formation for Enhanced Cartilage
434 Repair, *Ann. Biomed. Eng.* (2019). <https://doi.org/10.1007/s10439-019-02348-4>.

- 435 [14] J. Leijten, L.S. Moreira Teixeira, J. Bolander, W. Ji, B. Vanspauwen, J. Lammertyn, J. Schrooten,
436 F.P. Luyten, Bioinspired seeding of biomaterials using three dimensional microtissues induces
437 chondrogenic stem cell differentiation and cartilage formation under growth factor free
438 conditions, *Sci. Rep.* 6 (2016) 1–12. <https://doi.org/10.1038/srep36011>.
- 439 [15] G.D. DuRaine, W.E. Brown, J.C. Hu, K.A. Athanasiou, Emergence of Scaffold-Free Approaches
440 for Tissue Engineering Musculoskeletal Cartilages, *Ann. Biomed. Eng.* 43 (2015) 543–554.
441 <https://doi.org/10.1007/s10439-014-1161-y>.
- 442 [16] M. Sarem, O. Otto, S. Tanaka, V.P. Shastri, Cell number in mesenchymal stem cell aggregates
443 dictates cell stiffness and chondrogenesis, *Stem Cell Res. Ther.* 10 (2019) 10.
444 <https://doi.org/10.1186/s13287-018-1103-y>.
- 445 [17] M. Lehmann, F. Martin, K. Mannigel, K. Kaltschmidt, U. Sack, U. Anderer, Three-dimensional
446 scaffold-free fusion culture: The way to enhance chondrogenesis of in vitro propagated
447 human articular chondrocytes, *Eur. J. Histochem.* 57 (2013) 206–216.
448 <https://doi.org/10.4081/ejh.2013.e31>.
- 449 [18] R. Burdis, D.J. Kelly, Biofabrication and bioprinting using cellular aggregates, microtissues and
450 organoids for the engineering of musculoskeletal tissues, *Acta Biomater.* 126 (2021) 1–14.
451 <https://doi.org/10.1016/j.actbio.2021.03.016>.
- 452 [19] S. Bhumiratana, R.E. Eton, S.R. Oungouljian, L.Q. Wan, G.A. Ateshian, G. Vunjak-Novakovic,
453 Large, stratified, and mechanically functional human cartilage grown in vitro by mesenchymal
454 condensation, *Proc. Natl. Acad. Sci.* 111 (2014) 6940–6945.
455 <https://doi.org/10.1073/pnas.1324050111>.
- 456 [20] K. Futrega, J.S. Palmer, M. Kinney, W.B. Lott, M.D. Ungrin, P.W. Zandstra, M.R. Doran, The
457 microwell-mesh: A novel device and protocol for the high throughput manufacturing of
458 cartilage microtissues, *Biomaterials.* 62 (2015) 1–12.
459 <https://doi.org/10.1016/j.biomaterials.2015.05.013>.
- 460 [21] B.K. Babur, K. Futrega, W.B. Lott, T.J. Klein, J. Cooper-White, M.R. Doran, High-throughput
461 bone and cartilage micropellet manufacture, followed by assembly of micropellets into
462 biphasic osteochondral tissue, *Cell Tissue Res.* 361 (2015) 755–768.
463 <https://doi.org/10.1007/s00441-015-2159-y>.
- 464 [22] G.N. Hall, W.L. Tam, K.S. Andrikopoulos, L. Casas-Fraile, G.A. Voyiatzis, L. Geris, F.P. Luyten, I.
465 Papantoniou, Patterned, organoid-based cartilaginous implants exhibit zone specific
466 functionality forming osteochondral-like tissues in vivo, *Biomaterials.* 273 (2021) 120820.
467 <https://doi.org/10.1016/j.biomaterials.2021.120820>.
- 468 [23] R.Z. Lin, H.Y. Chang, Recent advances in three-dimensional multicellular spheroid culture for
469 biomedical research, *Biotechnol. J.* 3 (2008) 1172–1184.
470 <https://doi.org/10.1002/biot.200700228>.
- 471 [24] J.M. Karp, J. Yeh, G. Eng, J. Fukuda, J. Blumling, K.Y. Suh, J. Cheng, A. Mahdavi, J. Borenstein,
472 R. Langer, A. Khademhosseini, Controlling size, shape and homogeneity of embryoid bodies
473 using poly(ethylene glycol) microwells, *Lab Chip.* 7 (2007) 786–794.
474 <https://doi.org/10.1039/b705085m>.
- 475 [25] H. Tekin, M. Anaya, M.D. Brigham, C. Nauman, R. Langer, A. Khademhosseini, Stimuli-
476 responsive microwells for formation and retrieval of cell aggregates, *Lab Chip.* 10 (2010)
477 2411–2418. <https://doi.org/10.1039/c004732e>.

- 478 [26] B.C. Kim, J.H. Kim, H.J. An, W. Byun, J.H. Park, I.K. Kwon, J.S. Kim, Y.S. Hwang, Microwell-
479 mediated micro cartilage-like tissue formation of adipose-derived stem cell, *Macromol. Res.*
480 22 (2014) 287–296. <https://doi.org/10.1007/s13233-014-2044-7>.
- 481 [27] G.S. Jeong, Y. Jun, J.H. Song, S.H. Shin, S.H. Lee, Meniscus induced self organization of
482 multiple deep concave wells in a microchannel for embryoid bodies generation, *Lab Chip*. 12
483 (2012) 159–166. <https://doi.org/10.1039/c1lc20619b>.
- 484 [28] L. De Moor, S. Fernandez, C. Vercruyse, L. Tytgat, M. Asadian, N. De Geyter, S. Van
485 Vlierberghe, P. Dubruel, H. Declercq, Hybrid Bioprinting of Chondrogenically Induced Human
486 Mesenchymal Stem Cell Spheroids, *Front. Bioeng. Biotechnol.* 8 (2020) 1–20.
487 <https://doi.org/10.3389/fbioe.2020.00484>.
- 488 [29] L.S. Moreira Teixeira, J.C.H. Leijten, J. Sobral, R. Jin, A.A. van Apeldoorn, J. Feijen, C. van
489 Blitterswijk, P.J. Dijkstra, M. Karperien, High throughput generated micro-aggregates of
490 chondrocytes stimulate cartilage formation in vitro and in vivo, *Eur. Cells Mater.* 23 (2012)
491 387–399. <https://doi.org/10.22203/eCM.v023a30>.
- 492 [30] S.S. Ho, B.P. Hung, N. Heyrani, M.A. Lee, J.K. Leach, Hypoxic Preconditioning of Mesenchymal
493 Stem Cells with Subsequent Spheroid Formation Accelerates Repair of Segmental Bone
494 Defects, *Stem Cells*. 36 (2018) 1393–1403. <https://doi.org/10.1002/stem.2853>.
- 495 [31] A.P. Napolitano, D.M. Dean, A.J. Man, J. Youssef, D.N. Ho, A.P. Rago, M.P. Lech, J.R. Morgan,
496 Scaffold-free three-dimensional cell culture utilizing micromolded nonadhesive hydrogels,
497 *Biotechniques*. 43 (2007) 494–500. <https://doi.org/10.2144/000112591>.
- 498 [32] L. De Moor, I. Merovci, S. Baetens, J. Verstraeten, P. Kowalska, D. V. Krysko, W.H. De Vos, H.
499 Declercq, High-throughput fabrication of vascularized spheroids for bioprinting,
500 *Biofabrication*. 10 (2018) 35009. <https://doi.org/10.1088/1758-5090/aac7e6>.
- 501 [33] L. Zhao, S. Mok, C. Moraes, Micropocket hydrogel devices for all-in-one formation, assembly,
502 and analysis of aggregate-based tissues, *Biofabrication*. (2019).
503 <https://doi.org/10.1088/1758-5090/ab30b4>.
- 504 [34] V. Mironov, R.P. Visconti, V. Kasyanov, G. Forgacs, C.J. Drake, R.R. Markwald, Organ printing:
505 Tissue spheroids as building blocks, *Biomaterials*. 30 (2009) 2164–2174.
506 <https://doi.org/10.1016/j.biomaterials.2008.12.084>.
- 507 [35] C.M. McLeod, R.L. Mauck, On the origin and impact of mesenchymal stem cell heterogeneity:
508 New insights and emerging tools for single cell analysis, *Eur. Cells Mater.* 34 (2017) 217–231.
509 <https://doi.org/10.22203/eCM.v034a14>.
- 510 [36] J. van der Stok, M.K.E. Koolen, H. Jahr, N. Kops, J.H. Waarsing, H. Weinans, O.P. van der Jagt,
511 Chondrogenically differentiated mesenchymal stromal cell pellets stimulate endochondral
512 bone regeneration in critical-sized bone defects, *Eur. Cells Mater.* 27 (2014) 137–148.
513 <https://doi.org/10.22203/eCM.v027a11>.
- 514 [37] R. Burdis, D.J. Kelly, Biofabrication and bioprinting using cellular aggregates, microtissues and
515 organoids for the engineering of musculoskeletal tissues, *Acta Biomater.* 126 (2021) 1–14.
516 <https://doi.org/10.1016/j.actbio.2021.03.016>.
- 517 [38] A. Yamasaki, Y. Kunitomi, D. Murata, T. Sunaga, T. Kuramoto, T. Sogawa, K. Misumi,
518 Osteochondral regeneration using constructs of mesenchymal stem cells made by bio three-
519 dimensional printing in mini-pigs, *J. Orthop. Res.* 37 (2019) 1398–1408.
520 <https://doi.org/10.1002/jor.24206>.

- 521 [39] A.M. McDermott, S. Herberg, D.E. Mason, J.M. Collins, H.B. Pearson, J.H. Dawahare, R. Tang,
522 A.N. Patwa, M.W. Grinstaff, D.J. Kelly, E. Alsberg, J.D. Boerckel, Recapitulating bone
523 development through engineered mesenchymal condensations and mechanical cues for
524 tissue regeneration, *Sci. Transl. Med.* 11 (2019) 1–16.
525 <https://doi.org/10.1126/scitranslmed.aav7756>.
- 526 [40] D. Murata, S. Akieda, K. Misumi, K. Nakayama, Osteochondral Regeneration with a Scaffold-
527 Free Three-Dimensional Construct of Adipose Tissue-Derived Mesenchymal Stromal Cells in
528 Pigs, *Tissue Eng. Regen. Med.* 15 (2018) 101–113. [https://doi.org/10.1007/s13770-017-0091-](https://doi.org/10.1007/s13770-017-0091-9)
529 9.
- 530 [41] A. Yamasaki, Y. Kunitomi, D. Murata, T. Sunaga, T. Kuramoto, T. Sogawa, K. Misumi,
531 Osteochondral regeneration using constructs of mesenchymal stem cells made by bio three-
532 dimensional printing in mini-pigs, *J. Orthop. Res.* 37 (2019) 1398–1408.
533 <https://doi.org/10.1002/jor.24206>.
- 534 [42] W. Kafienah, T.J. Sims, *Biochemical Methods for the Analysis of Tissue-Engineered Cartilage*,
535 in: *Biopolym. Methods Tissue Eng.*, Humana Press, New Jersey, 2004: pp. 217–230.
536 <https://doi.org/10.1385/1-59259-428-X:217>.
- 537 [43] X. Tang, L. Fan, M. Pei, L. Zeng, Z. Ge, Evolving concepts of chondrogenic differentiation:
538 History, state-of-the-art and future perspectives, *Eur. Cells Mater.* 30 (2015) 12–27.
539 <https://doi.org/10.22203/eCM.v030a02>.
- 540 [44] R.A. Somoza, J.F. Welter, D. Correa, A.I. Caplan, Chondrogenic differentiation of mesenchymal
541 stem cells: Challenges and unfulfilled expectations, *Tissue Eng. - Part B Rev.* 20 (2014) 596–
542 608. <https://doi.org/10.1089/ten.teb.2013.0771>.
- 543 [45] L.A. Solchaga, K. Penick, J.D. Porter, V.M. Goldberg, A.I. Caplan, J.F. Welter, FGF-2 enhances
544 the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal
545 stem cells, *J. Cell. Physiol.* 203 (2005) 398–409. <https://doi.org/10.1002/jcp.20238>.
- 546 [46] F. Ng, S. Boucher, S. Koh, K.S.R. Sastry, L. Chase, U. Lakshmipathy, C. Choong, Z. Yang, M.C.
547 Vemuri, M.S. Rao, V. Tanavde, PDGF, *tgf- 2*. And FGF signaling is important for differentiation
548 and growth of mesenchymal stem cells (mscs): Transcriptional profiling can identify markers
549 and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic,
550 and ost, *Blood.* 112 (2008) 295–307. <https://doi.org/10.1182/blood-2007-07-103697>.
- 551 [47] C.A. Hellingman, W. Koevoet, N. Kops, E. Farrell, H. Jahr, W. Liu, R.J.B. De Jong, D.A. Frenz,
552 G.J.V.M. Van Osch, Fibroblast growth factor receptors in in vitro and in vivo chondrogenesis:
553 Relating tissue engineering using adult mesenchymal stem cells to embryonic development,
554 *Tissue Eng. - Part A.* 16 (2010) 545–556. <https://doi.org/10.1089/ten.tea.2008.0551>.
- 555 [48] S. Boeuf, W. Richter, Chondrogenesis of mesenchymal stem cells: Role of tissue source and
556 inducing factors, *Stem Cell Res. Ther.* 1 (2010) 1–9. <https://doi.org/10.1186/scrt31>.
- 557 [49] L. Longobardi, L. O’Rear, S. Aakula, B. Johnstone, K. Shimer, A. Chytil, W.A. Horton, H.L.
558 Moses, A. Spagnoli, Effect of IGF-I in the chondrogenesis of bone marrow mesenchymal stem
559 cells in the presence or absence of TGF- β signaling, *J. Bone Miner. Res.* 21 (2006) 626–636.
560 <https://doi.org/10.1359/jbmr.051213>.
- 561 [50] M. Ulrich-Vinther, M.D. Maloney, E.M. Schwarz, R. Rosier, R.J. O’Keefe, Articular Cartilage
562 Biology, *J. Am. Acad. Orthop. Surg.* 11 (2003) 421–430. [https://doi.org/10.5435/00124635-](https://doi.org/10.5435/00124635-200311000-00006)
563 200311000-00006.

- 564 [51] C. Wernecke, H.J. Braun, J.L. Dragoo, The effect of intra-articular corticosteroids on articular
565 cartilage: A systematic review, *Orthop. J. Sport. Med.* 3 (2015) 1–7.
566 <https://doi.org/10.1177/2325967115581163>.
- 567 [52] B. Johnstone, T.M. Hering, A.I. Caplan, V.M. Goldberg, J.U. Yoo, In Vitro Chondrogenesis of
568 Bone Marrow-Derived Mesenchymal Progenitor Cells, *Exp. Cell Res.* 238 (1998) 265–272.
569 <https://doi.org/10.1006/excr.1997.3858>.
- 570 [53] T. Li, B. Liu, K. Chen, Y. Lou, Y. Jiang, D. Zhang, Small molecule compounds promote the
571 proliferation of chondrocytes and chondrogenic differentiation of stem cells in cartilage
572 tissue engineering, *Biomed. Pharmacother.* 131 (2020) 110652.
573 <https://doi.org/10.1016/j.biopha.2020.110652>.
- 574 [54] A. Derfoul, G.L. Perkins, D.J. Hall, R.S. Tuan, Glucocorticoids Promote Chondrogenic
575 Differentiation of Adult Human Mesenchymal Stem Cells by Enhancing Expression of Cartilage
576 Extracellular Matrix Genes, *Stem Cells.* 24 (2006) 1487–1495.
577 <https://doi.org/10.1634/stemcells.2005-0415>.
- 578 [55] L.R. Devireddy, M. Myers, R. Screven, Z. Liu, L. Boxer, A serum-free medium formulation
579 efficiently supports isolation and propagation of canine adipose-derived mesenchymal
580 stem/stromal cells, *PLoS One.* 14 (2019) 1–21.
581 <https://doi.org/10.1371/journal.pone.0210250>.
- 582 [56] N.N. Shipunova, N.A. Petinati, N.I. Drize, Effect of hydrocortisone on multipotent human
583 mesenchymal stromal cells, *Bull. Exp. Biol. Med.* 155 (2013) 159–163.
584 <https://doi.org/10.1007/s10517-013-2102-8>.
- 585 [57] J. Wang, D. Elewaut, I. Hoffman, E.M. Veys, G. Verbruggen, Physiological levels of
586 hydrocortisone maintain an optimal chondrocyte extracellular matrix metabolism, *Ann.*
587 *Rheum. Dis.* 63 (2004) 61–66. <https://doi.org/10.1136/ard.2002.005298>.
- 588 [58] Y. Mifune, T. Matsumoto, S. Murasawa, A. Kawamoto, R. Kuroda, T. Shoji, T. Kuroda, T. Fukui,
589 Y. Kawakami, M. Kurosaka, T. Asahara, Therapeutic superiority for cartilage repair by CD271-
590 positive marrow stromal cell transplantation, *Cell Transplant.* 22 (2013) 1201–1211.
591 <https://doi.org/10.3727/096368912X657378>.
- 592 [59] L. Jiang, A. Ma, L. Song, Y. Hu, H. Dun, P. Daloz, Y. Yu, J. Jiang, M. Zafarullah, H. Chen,
593 Cartilage regeneration by selected chondrogenic clonal mesenchymal stem cells in the
594 collagenase-induced monkey osteoarthritis model, *J. Tissue Eng. Regen. Med.* 8 (2014) 896–
595 905. <https://doi.org/10.1002/term.1676>.
- 596