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1 **Combining biomimetic collagen/hyaluronan hydrogels with**
2 **discogenic growth factors promotes mesenchymal stroma cell**
3 **differentiation into Nucleus Pulposus like cells**

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Abstract

Based on stem cell injection into degenerated Nucleus Pulposus (NP), novel treatments for intervertebral disc (IVD) regeneration were disappointing because of cell leakage or inappropriate cell differentiation. In this study, we hypothesized that mesenchymal stroma cells encapsulated within injectable hydrogels possessing adequate physico-chemical properties would differentiate into NP like cells. Composite hydrogels consisting of type I collagen and tyramine-substituted hyaluronic acid (THA) were formed to mimic the NP physico-chemical properties. Human bone marrow derived mesenchymal stroma cells (BM-MSCs) were encapsulated within hydrogels and cultivated in proliferation medium (supplemented with 10% fetal bovine serum) or differentiation medium (supplemented with GDF5 and TGF β 1) over 28 days. Unlike pure collagen, collagen/THA composite hydrogels were stable over 28 days in culture. In proliferation medium, cell viability within pure collagen hydrogels was high whereas that in composite and pure THA hydrogels was lower due to the weaker cell adhesion. Nonetheless, BM-MSCs proliferated in all hydrogels. In composite hydrogels, cells exhibited a rounded morphology similar to NP cells. The differentiation medium did not impact hydrogel stability, cell morphology but negatively impact cell viability in pure collagen hydrogels. A high THA content within hydrogels promoted the gene expression of NP markers such as collagen II, aggrecan, SOX9 and Cytokeratin 18 at day 28. The differentiation medium potentialized this effect with an earlier and higher expression of these NP markers. Taken together, these results show that physico-chemical properties of collagen/THA composite hydrogels and GDF5/TGF β 1 act in synergy to promote the differentiation of BM-MSCs into NP like cells.

Keywords: collagen, tyramine-substituted hyaluronic acid, intervertebral disc regeneration, composite hydrogels, mesenchymal stroma cells, physico-chemical properties, discogenic factors.

77 1. Introduction

78

79 Low back pain (LBP) is a leading cause of disability worldwide which has a substantial socioeconomic
80 impact and poses a burden on healthcare ¹. LBP is often attributed to intervertebral disc (IVD)
81 degeneration which leads to a variety of spinal problems such as disc herniation ². The nucleus
82 pulposus (NP) is predominantly affected during the early stages of degeneration ³. This centrally
83 located tissue is highly hydrated (80% wet weight) and composed of proteoglycans and collagen II
84 which allow for a viscoelastic behavior that confers shock-absorbing properties within the IVD ⁴. NP
85 degeneration involves alterations in cell function, cell death and modification of matrix composition,
86 ultimately resulting in a loss of disc height and spinal instability ^{5, 6}.

87 The standard treatments for IVD degeneration involve conservative options like medication and
88 physical therapy. When these treatments are not efficient enough and the pain is intolerable, invasive
89 surgeries such as spinal fusion or disc arthroplasty are required. Surgeries can have significant
90 complications and are suspected to accelerate degeneration of adjacent IVDs ^{7,8}. Since the 00's, several
91 alternative treatments have emerged. Among them, cell therapy based on the injection of
92 mesenchymal stroma cells (MSCs) within degenerated Nucleus Pulposus has been evaluated ⁹.
93 Unfortunately, the reported outcomes are disappointing due to cell leakage and/or incomplete cell
94 differentiation ¹⁰. Nowadays, a consensus exists on the interest to encapsulate stem cells within
95 hydrogels. After injection, such systems should allow for better cell retention, survival, and
96 differentiation into NP-like cells ¹⁰⁻¹². Injectable hydrogels have been found to be optimal candidates
97 as cell carrier due to their minimally invasive delivery method. Hydrogels can be synthesized from
98 synthetic polymers, natural polymers or extracellular matrix (ECM) blends. Synthetic polymers are
99 appropriate to reach mechanical and physical properties mimicking the NP ones ^{13,14}. However, these
100 materials lack of patterns for cellular recognition. Hence, they are usually functionalized to optimize
101 cell survival. Poly(N-isopropylacrylamide (pNIPAM) has been evaluated in vitro to treat the
102 degenerated NP. Encapsulated MSCs can differentiate within pNIPAM hydrogels but this polymer is
103 not degraded by cells ¹⁵. Polyethylene glycol (PEG) has been broadly used either on its own or
104 functionalized with Laminin ^{4,16}. Despite its high cytocompatibility, the degradation rate is very low,
105 thereby slowing down the hydrogel remodeling by encapsulated cells ^{16,17}.

106 Some natural polymers such as fibrin, collagens and hyaluronan allow cell adhesion but they usually
107 exhibit insufficient mechanical properties ^{14, 18}. To circumvent this issue, biopolymers have to be
108 crosslinked ⁴.

109 Collagen is a major component of the natural Nucleus Pulposus extracellular matrix (ECM) as it
110 provides its fibrillar structure. In addition, this protein is biodegradable, biocompatible and allows for
111 cell adhesion and proliferation. This is the reason why this protein was one of the first studied for disc

112 regeneration ¹⁹. However, non-crosslinked collagen hydrogels have poor mechanical strength, high
113 enzymatic degradation, and weak stability after cell encapsulation. To overcome these limitations,
114 collagen is often combined with other polymers ^{12, 20}. Hyaluronic acid (HA) is a non-sulfated
115 glycosaminoglycan (GAG) that is richly present in the IVD (10% dry weight). Besides proteoglycans, HA
116 participates to NP hydration and has therefore been broadly studied for IVD regeneration. However,
117 HA does not form hydrogels on its own. It has to be functionalized and crosslinked via chemicals,
118 enzymatic methods or photo initiators ²¹. Moreover, HA does not allow a high cell adhesion despite
119 the presence of its specific cell receptors such as CD 44 and RHAMM ^{22,23}.

120 Combining collagen and HA to form a hybrid hydrogel allows to maintain the biological benefits of
121 collagen while improving the hydrogel mechanical properties. Unfortunately, these hydrogels require
122 crosslinking with toxic chemicals such as EDC/NHS. In addition, such crosslinking inhibits collagen
123 fibrillogenesis and the resulting structure does not mimic the natural NP topography. Fabricating
124 composite hydrogels composed of an interpenetrating network of HA and fibrillar collagen is of high
125 interest for IVD regeneration but difficult to obtain due to the strong collagen/HA interaction which
126 leads to precipitation ²⁴. So, a first gelation of a biopolymer is required before integrating the second
127 one. In this case, mechanical properties are not improved and some biopolymer leakage issues are
128 observable ²⁵. In addition, these formulations are barely injectable.

129 We recently demonstrated a new method for synthesizing biomimetic collagen/HA composite
130 hydrogels that maintains collagen fibrils while providing mechanical strength and hydration ²⁶. This
131 "one pot" approach is based on the co-gelation of pre-neutralized type I collagen (to trigger collagen
132 gelling) with a 6% substituted tyramine-conjugated hyaluronic acid (THA) using horse radish peroxidase
133 (HRP) and hydrogen peroxide (H₂O₂) as crosslinker. Collagen I was used in this study because of its
134 availability and its ability to differentiate MSCs into NP like cells ¹⁹. The kinetics of gelation were tuned
135 to generate collagen fibrils before THA gelling. We performed a systematic study to understand the
136 impact of the THA content on the hydrogel properties. Composite hydrogels with a high THA content
137 had mechanical properties close to those of NP, were highly hydrated and more resistant against
138 enzymatic degradation. Last, they allowed for fibroblast survival and proliferation thanks to the mild
139 conditions of fabrication via enzymatic THA crosslinking ²⁶.

140 In the current study, we hypothesized that the physicochemical properties of the previously developed
141 Type I collagen/THA composite hydrogels could support bone marrow derived mesenchymal stroma
142 cells (BM-MS) survival and differentiation into NP-like cells. To this end, we first synthesized injectable
143 composite hydrogels that closely mimic the hydration, mechanical properties, and biochemical
144 composition of the Nucleus Pulposus. Then, we evaluated the effect of the THA content on the cell
145 behavior. Last, we explored the ability of two pro-discogenic growth factors, *i.e* GDF5 (growth

146 differentiation factor 5) and TGF-B1 (transforming growth factor beta1) to synergically promote BM-
147 MSC differentiation.

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149 **2. Materials and Methods**

150

151 2.1 Collagen extraction and purification

152

153 Type I collagen was extracted from rat tails of Young Wistar males as previously described (Approval
154 number IJM B751317 from the Ministère de l'Agriculture)²⁷. Briefly, rat tails were rinsed with 70%
155 ethanol, placed in a safety cabinet and cut in 1 cm sections from their extremity to extract tendons.
156 After several rinses in 1X PBS (Thermo Scientific), tendons were solubilized in 500 mM acetic acid (Carlo
157 Erba, France) for 24 hours. Then, collagen was purified by precipitation using 0.7 M NaCl (Merck).
158 Precipitates were dissolved in a fresh 500 mM acetic acid solution. Salt elimination and pH adjustment
159 at 4.5 was performed by dialysis against 17 mM acetic acid. The final solution was centrifuged at 30000
160 g for 4 hours to remove aggregates and supernatant was stored at 4°C until further use. The collagen
161 concentration was determined by hydroxyproline titration²⁸ and purity assessed by SDS-PAGE
162 electrophoresis (MiniProtean TGX, Biorad). Finally, the concentration was set to 8.8mg.mL⁻¹ via
163 evaporation under a safety cabinet and used as collagen stock solution.

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166 2.2 Hyaluronan conjugation with tyramine

167 Conjugation of hyaluronan (HA) (MW: 280 kDa, Contipro Biotech s.r.o, Contipro, product number: 639-
168 80-01) with tyramine was performed by amidation reaction between HA carboxylic groups and the
169 amine group of Tyramine Hydrochloride (Sigma Aldrich). Hyaluronic acid sodium salt (2 g, 5 mmol
170 carboxylic groups) was dissolved in 200 mL of ultrapure water at a final concentration of 1% (w/v)
171 overnight. The following day, the HA solution was warmed up to 37 °C using a thermostatic oil bath.
172 Five mmol of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) were
173 added to the HA solution. Subsequently, 5 mmol of Tyr were dissolved in ca. 5 mL deionized H₂O and
174 added dropwise. The whole mixture was stirred at 37 °C for 24 h. Following the addition of 32 mL of a
175 NaCl saturated solution and a 30 min stirring, the newly formed tyramine-HA conjugate (THA) was
176 precipitated by adding dropwise 96% alcohol. After several washes in 96% alcohol, the precipitate was
177 collected by filtration under vacuum with a Gooch filter P3 and dried at 40 °C for 48h. Last, THA was
178 dissolved in 1X PBS and its concentration adjusted at 6% (w/v) to obtain a THA stock solution. 0.1 M
179 silver nitrate was used to detect salt residues. Synthesized THA conjugates were characterized using

180 UV-vis spectroscopy as previously reported^{26,29}. The molar degree of substitution (DS_{mol}, %) was 6%,
181 calculated by measuring the absorbance at 275 nm of a 0.1% (w/v) THA solution in ultrapure water
182 using a Cary 5000 UV-Vis-NIR Spectrophotometer (Agilent Technologies).

183

184 2.3 Synthesis of Collagen/THA composite hydrogels

185

186 Collagen concentration was kept constant at 0.4% (w/v) for all hydrogels and varied amounts of THA
187 were added to obtain collagen/THA ratios – 1:2 and 1:5 with final THA concentrations of 0.8% and 2%
188 (w/v) respectively. Pure collagen (0.4% w/v) and THA (2% w/v) hydrogels were used as controls (Figure
189 1). For a 1 mL hydrogel, collagen gelling was triggered by pH increase to 7 using 100 µL of 10X-PBS and
190 40 µL of 0.1 M NaOH. Additionally, THA gelling was carried by 0.5 U.mL⁻¹ HRP (Horse Raddish
191 Peroxidase, Merck) and 0.6mM H₂O₂ (Merck, France). Typically, collagen/THA hydrogels were
192 performed in a 12 well plate by first mixing THA, HRP, 10X PBS and 0.1 M NaOH. After an hour
193 incubation in ice, collagen was added and THA gelling triggered by H₂O₂ addition. Last, hydrogels were
194 placed in an incubator at 37°C for 30 min to complete the gelation process. The different volumes to
195 generate the different composite hydrogels are listed in Supplementary Information N°1.

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198 2.4 Human bone marrow mesenchymal stroma cells (hBM-MSC) culture

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200 All experiments with hBM-MSC were performed in accordance with the guidelines of Lariboisière
201 Hospital and experiments were approved by Paris Cité University. Human BM-MSCs were isolated from
202 bone marrow samples obtained as discarded tissue during orthopaedic surgeries at Trousseau and
203 Lariboisière Hospital (Paris, France) following Lariboisiere Hospital regulations and after patient's or
204 parent's informed consent (4 donors, 5-22 years old, 1 female, 3 males). The cells were isolated by
205 plastic adhesion, amplified and analyzed using flow cytometry to ensure expression of characteristic
206 MSC markers (CD90+, CD73+, CD105+, CD45-). BM-MSCs from each donor were amplified separately
207 in Alpha MEM Eagle (PANTMBIOTECH) supplemented with 10% fetal bovine serum (FBS) (Dominique
208 Dutscher) and 1% Penicillin/Streptomycin (Gibco). Cells were cultivated in incubator set to 37°C and
209 5% CO₂ with culture medium changed every 3 days. When reaching 85-90%, BM-MSCs were used at
210 passage 5 for 3D culture.

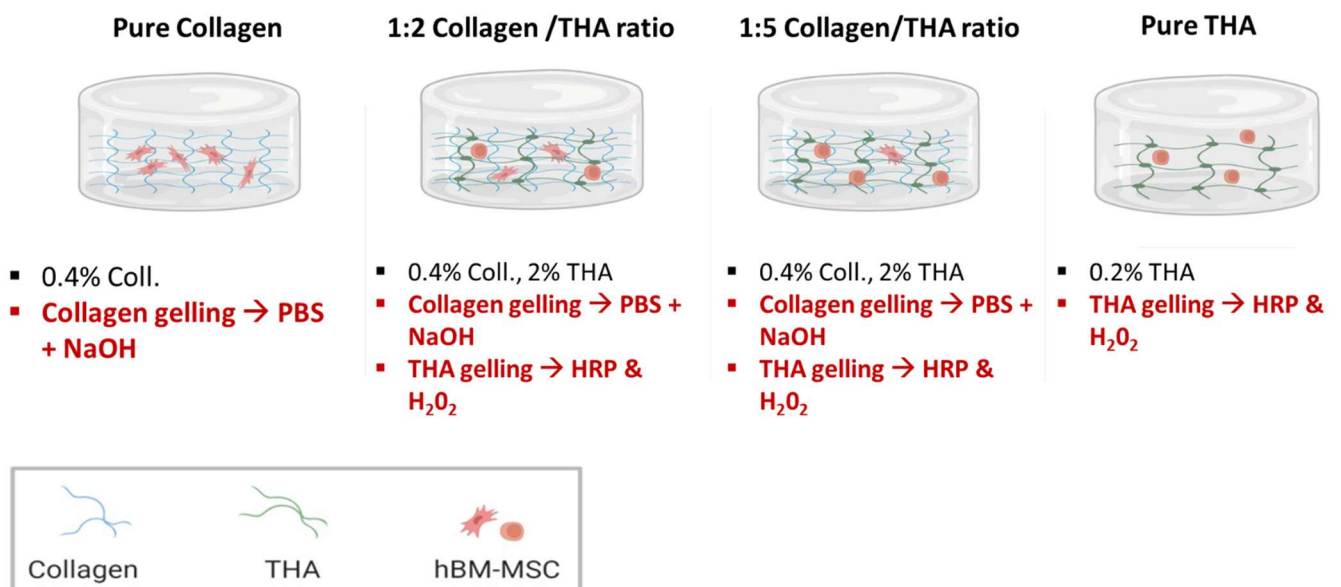
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213 2.5 Cell encapsulation within collagen/THA composite hydrogels and 3D cell culture

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Prior to their encapsulation, BM-MSCs from each donor were detached with 0.05% trypsin, counted, pooled at 1:4 and their final density adjusted to 5×10^6 cells.mL⁻¹. Pure and composite hydrogels were carried out following the same procedure than that described in section 2.3 (Figure 1). Working on ice, 100 μ L of cell suspension was then mixed with each forming hydrogel, just after the H₂O₂ addition. The resulting cell density was about 500,000 cells per mL. The solution was dispensed in 12-well culture plates and incubated for 30 mins at 37°C to ensure complete gelation. Then, 2 mL of culture medium was added and hydrogels were cultivated over 28 days in an incubator set to 37°C and 5% CO₂. Two different culture media were used: proliferation and differentiation medium. The proliferation medium consisted of DMEM (4.5g.L⁻¹ glucose with pyruvate and Glutamax) (Gibco), 10% FBS, 1% penicillin/streptomycin and 1% amphotericin B (Gibco). The differentiation medium was a serum free DMEM medium (4.5g.L⁻¹ glucose with pyruvate and Glutamax) with 1% penicillin/streptomycin and 1% amphotericin B supplemented with ITSTM + Premix (Corning), 50uM ascorbic acid (Merck), 10⁻⁸M dexamethasone (Merck), 10ng.ml⁻¹ human TGF- β 1 (Peprotech) and 100ng.ml⁻¹ human GDF-5 (Peprotech). Media were changed every 3 days and analysis performed after 1, 7, 14 and 28 days.



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Figure 1: Different formulations of collagen/THA hydrogels

2.6 Measurement of collagen/THA composite hydrogel contraction

236 Gross images of hydrogels were captured at each time point using a numeric camera (Keence) to
237 qualitatively assess potential change in area due to cell contraction. These images were further
238 analyzed via ImageJ[®] software to deduce surface area of the constructs which was expressed as a
239 percentage of the original area i.e., 3.8cm² for a 12-well plate. Six hydrogels for each condition were
240 measured.

241

242 2.7 Scanning electron microscopy

243 Hydrogels were fixed overnight at 4 °C using a 4% paraformaldehyde (PFA) solution (w/v) in PBS. This
244 step was followed by a 1 h fixation at 4 °C in a 2.5% glutaraldehyde solution diluted in 0.1 M cacodylate
245 buffer pH 7.2. The samples were then dehydrated using ethanol baths with increasing concentrations
246 up to 100% and then dried using a supercritical dryer. The samples were finally coated with a 20 nm
247 gold layer and observed using a Hitachi S-3400N Scanning Electron Microscope (operating at 10 kV).
248 For each sample, images were acquired at a X10000 magnification. Three samples per hydrogel
249 formulation were analyzed.

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252 2.8 Rheological measurements

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254 Shear oscillatory measurements were performed on hydrogels using an Anton Paar rheometer
255 MCR302 fitted with a 25 mm sand-blasted parallel plate upper geometry. All tests were performed at
256 20 °C with frequency sweeps. Mechanical spectra, namely storage G' and loss G'' moduli versus
257 frequency (0.01-10 Hz), were recorded at an imposed 1% strain, which corresponded to non-
258 destructive conditions, as previously checked with an amplitude sweep (data not shown). In order to
259 test all hydrogels in the same conditions, before each run, the gap between base and geometry was
260 chosen to apply a slight positive normal force on hydrogels during measurement: respectively 0.04 N
261 and 0.1 N. At least six samples per hydrogel formulation were tested.

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264 2.9 Injectability

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266 The different formulations were poured into a 1 mL syringe. The mixtures were injected through a 22G
267 needle into wells of a 12 well plate. After a 30 min incubation at 37°C to enable the hydrogel formation,
268 2 mL of culture medium was added on top of each hydrogel. Then, the mechanical properties were

269 measured by rheometry as described in section 2.8. Casted hydrogels from the different formulation
270 were used as controls.

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273 2.10 Cell metabolic activity

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275 Cell metabolic activity was monitored after 1, 7, 14 and 28 days of culture using Alamar Blue assay.
276 After 3 washes in DMEM without phenol red (Thermo Scientific), 3D cell-laden hydrogels were
277 incubated at 37°C with 300 μL of a 10 $\mu\text{g}\cdot\text{mL}^{-1}$ resazurin solution for 5 hours. For this purpose, a stock
278 solution of resazurin at 100 $\mu\text{g}\cdot\text{mL}^{-1}$ (Thermo Scientific) was diluted 1 in 10 in colorless DMEM culture
279 medium. The supernatant was then collected in each well, diluted with 500 μL of fresh colorless
280 medium, and the absorbance measured at $\lambda = 570 \text{ nm}$ and $\lambda = 600 \text{ nm}$ using a Varioskan™ LUX
281 multimode microplate reader (Thermofisher). The percentage of resazurin reduction was calculated
282 following the instructions provided by the supplier. BM-MSCs cultured in pure collagen hydrogels at
283 day 1 were used as controls and ascribed an arbitrary value of 100%. The results were expressed as a
284 percentage of reduced absorbance compared to the control. Four samples per condition and time
285 point were analyzed.

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287

288 2.11 Cell morphology

289

290 Morphology of BM-MSCs encapsulated within collagen/THA hydrogels was observed on histological
291 sections. Hydrogels were fixed in a 4% PFA solution (w/v) in PBS for 24 hours, then dehydrated with
292 ethanol baths of increasing concentration (24h in 70%, 3h in 95% and 3h in 100% EtOH). Afterwards,
293 they were left in butanol for 4 days and embedded in paraffin. Seven micrometer transverse sections
294 were performed using a microtome (Leica). Sections were rehydrated and stained with Mayer's
295 Hematoxylin solution (Merck) for 5 min. Then, sections were rinsed with deionized H_2O and
296 dehydrated again using ethanol and toluene. The sections were finally mounted between a glass and
297 coverslip using an Eukitt mounting medium. Samples were imaged at X400 magnification with a Nikon
298 Eclipse E600 POL equipped with a Nikon DS-Ri1 camera. Three samples per condition and time point
299 were analyzed.

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301

302 2.12 Indirect immunodetection

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304 Antigen retrieval was performed on rehydrated histological sections using citrate buffer (10mM
305 sodium citrate, 0.05% Tween 20, pH 6.0) at 95°C for 20 min. After cooling, sections were rinsed three
306 times in PBS and incubated in blocking solution (0.05% Tween PBS, 1% bovine serum albumin, 10%
307 horse serum) for one hour. After primary antibodies addition, each histological section was incubated
308 in a moist chamber at 4°C overnight. The monoclonal mouse anti human collagen 2 (AbCam, product
309 number: ab34712) was diluted 1 in 50 in blocking solution to obtain a concentration of 2 ng.mL⁻¹ .
310 Regarding the monoclonal rabbit antihuman aggrecan (Abcam, product number: ab186414), the stock
311 solution was diluted 1 in 100 in blocking solution to obtain a 1 mg.mL⁻¹ concentration. The following
312 day, the sections were rinsed three times in PBS for 5 min. Then, the Mouse and Rabbit Specific
313 HRP/DAB (ABC) detection IHC kit (AbCam) was used to reveal the collagen 2 or aggrecan labelling.
314 Briefly, a secondary antibody coupled with biotin was added, the endogenous peroxidases were
315 inhibited by H₂O₂ addition and the Steptavidin/HRP system was used to oxidize DAB into a brown
316 product, thereby allowing the antigen detection. Negative controls were carried out following the
317 same procedure but without incubation with the primary antibody. Sections were then counterstained
318 with the Mayer's Hematoxylin solution for 30 s and then extensively washed in deionized water. The
319 sections were finally dehydrated and mounted as previously described in section 2.10. Samples were
320 imaged at X100 and X400 magnification with a Nikon Eclipse E600 POL. Three samples per condition
321 and time point were analyzed.

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324 2.13 RNA purification

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326 Total RNA was extracted from cellularized hydrogels by homogenization in 1 mL of TRIzol® reagent
327 (Thermo Scientific) using an ULTRA-TURRAX® homogenizer. A first centrifugation step at 5000 g was
328 conducted for 15 min to remove debris. Phase separation was then performed by adding 0.2 mL of
329 chloroform (Gibco) and centrifuged at 10000 g for 15 min. After collection of the aqueous phase, RNA
330 purification was carried out using a RNeasy kit (Qiagen), according to the supplier's recommended
331 procedure. Last, RNA concentration and purity were determined by UV spectrophotometry using a
332 Varioskan LUX multi-plate reader (Thermo Scientific).

333

334

335 2.14 Reverse transcription into cDNA

336

337 1uL of random primers (200uM) (Invitrogen) and 1 µL deoxyribonucleosides triphosphates(10mM)
338 (Invitrogen) were added to 10ul RNA aliquots (around 300-500 ng). Following denaturation of

339 secondary structure at 65°C and primers binding, 5X reaction buffer, dithiothreitol (0.2M) and Moloney
340 murine leukemia virus (M-MLV) (Invitrogen) were added. After 60 min at 37°C and the reaction was
341 stopped by heating at 70°C for 10 min. The resulting cDNAs were stored at -20°C until further use.

342

343

344 2.15 Real Time PCR

345

346 Gene expression of COL2A1, ACAN, COL1A1, SOX9, IBSP, KRT18 and COL 10 was evaluated using
347 reverse transcription quantitative PCR (RT-qPCR) in a Light Cycler 480 system (Roche). For this
348 purpose, the Light Cycler FastStart DNA Master plus SYBR Green I kit (Roche) was used.
349 Appropriate primers for real time RT-PCR are listed in the Supplementary information N°2.
350 Cycling conditions were: initial Taq polymerase activation at 95°C for 5 min followed by 40 cycles,
351 each cycle consisting of 10 s denaturation at 95°C; 15 s annealing at 60°C and 15 s elongation at
352 72°C. Then, a melting curve was generated by increasing the temperature from 60°C to 97°C at a
353 rate of 0.1°C/s to assess the reaction specificity. The results were analyzed using a relative
354 quantification following the Pfaffl method³⁰. The efficiencies of the target and reference primer
355 pairs were measured by producing a standard curve based on the amplification of a serial dilution
356 of cDNAs. The mRNA transcript level of each target gene was normalized with the housekeeping
357 genes RPL13A-1 and GAPDH. Fold changes in gene expression were calculated for each target
358 gene relative to a calibration point which is the normalized gene expression of this target gene in
359 pure collagen hydrogels cultured in differentiation medium at day 1. The value 1 was arbitrary
360 given to this calibration point. Three samples precondition and per time point were analyzed.

361

362 2.16 Statistical analysis

363

364 All experiments were carried out at least twice, and the results were expressed as mean values +
365 standard deviation (SD). The differences between the different formulations were analyzed for each
366 time point using Kruskal-Wallis test. Then, a Dunn Test was used as post-hoc test to determine which
367 groups are different. A p value < 0.05 was considered significant.

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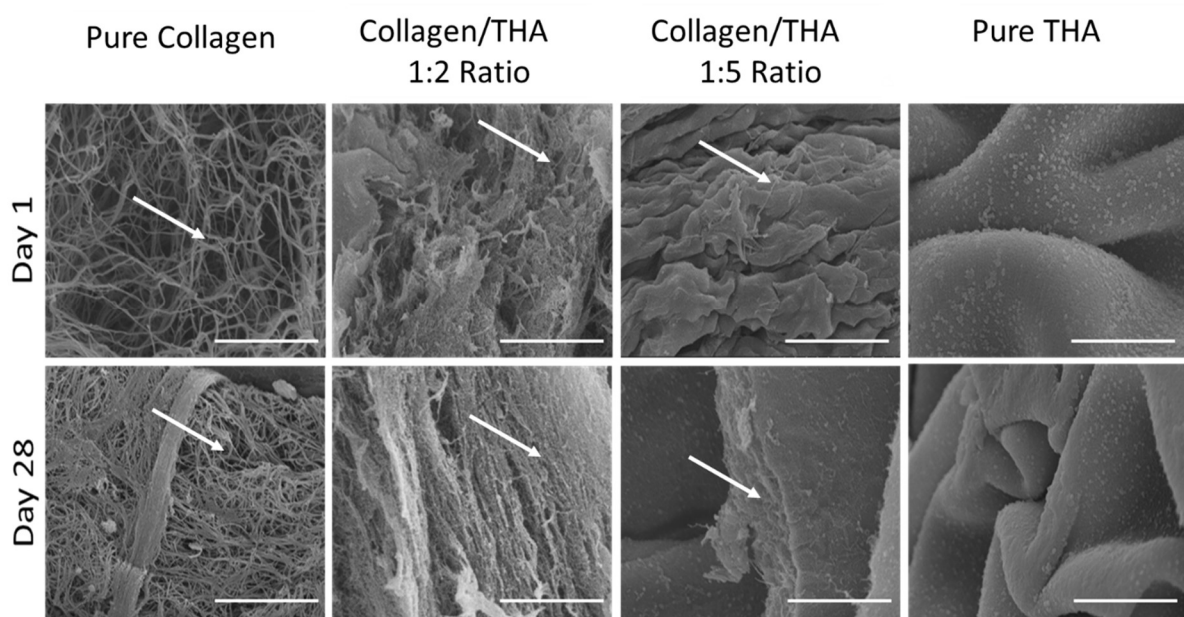
370 3. Results

371

372 3.1 Collagen/THA hydrogels ultrastructure

373 Collagen was maintained at a concentration of 0.4% for all hydrogel formulations. Cell-laden collagen
374 hydrogels exhibited a loose fibrillar network at day 1 characteristic of low-concentrated hydrogels
375 (Figure 2). As expected, their ultrastructure changed during the cell culture to become denser at day
376 28 due to hydrogel contraction mediated by cell activity²⁴. Indeed, acellular hydrogels did not exhibit
377 any structural changes after 28 days in culture medium (Electronic Supplementary Information N°3). A
378 mixture of 0.4% collagen and 0.8% THA (1:2 ratio) results in composite hydrogels showing aggregates
379 of collagen fibrils (Figure 2). The latter seemed to be packed by the THA presence. Interestingly, these
380 hydrogels were not modified during the cell culture experiment as similar structures were observed at
381 day 1 and 28. The addition of 2% THA (1:5 ratio) changed the collagen organization at day 1. Some
382 sheets-like morphological features reminiscent of the pure THA hydrogels were observed. However,
383 some collagen fibrils were still visible inside these sheets at day 28. Pure THA hydrogels exhibited a
384 characteristic sheet-like structure at day 1 and 28²⁶. Similar results were obtained with the
385 differentiation medium (GDF5 and TGFB1) did not impact the ultrastructure of hydrogels (Electronic
386 Supplementary Information N°4).

387



388

389 **Figure 2:** Ultrastructure of cellularized collagen/THA composite hydrogels observed by scanning
390 electron microscopy after 1 day (top) or 28 days (bottom) of culture in proliferation medium. Hydrogel
391 surface observed after hydrogel fracture. White arrows show collagen fibrils. Scale Bar: 5 μ m.

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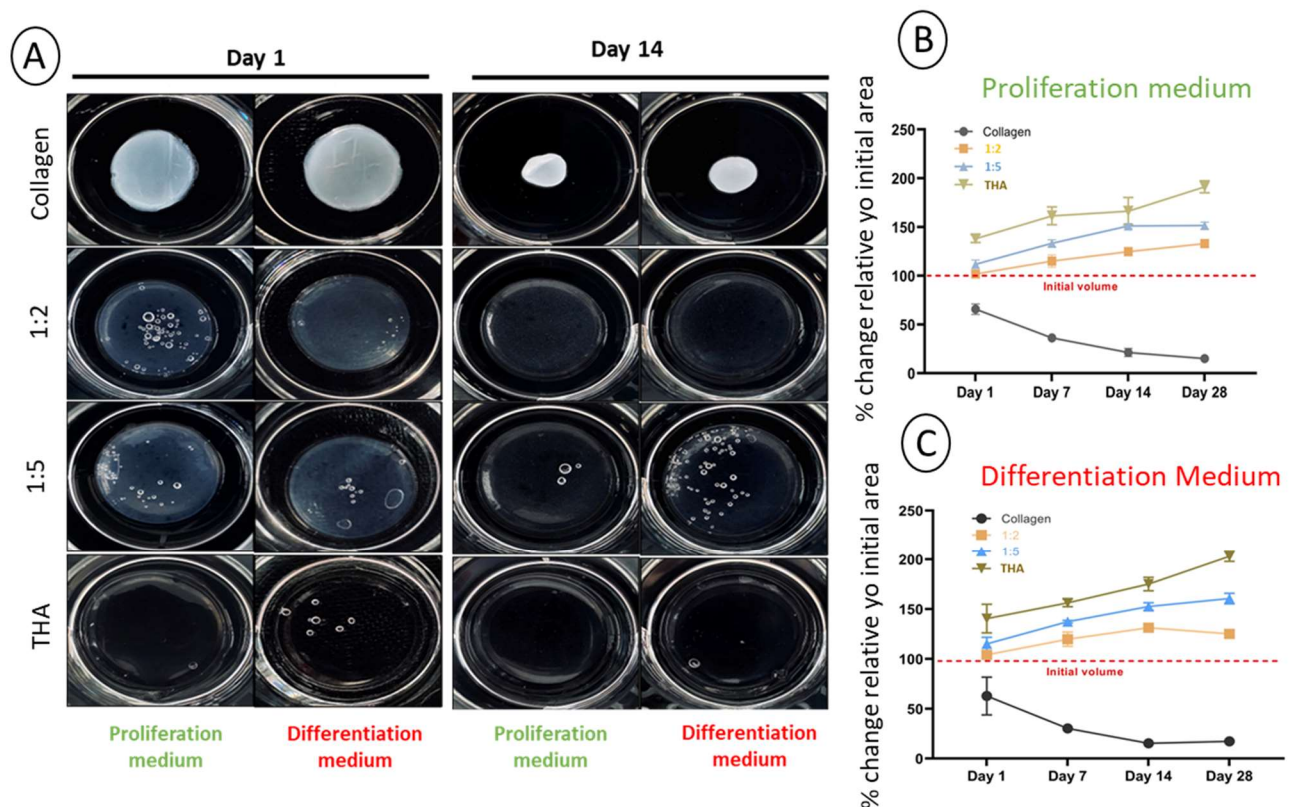
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395 3.2 Collagen/THA composite hydrogel stability

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397 The main parameter considered for assessing the stability of cell-laden hydrogels was the change in
398 surface area. With an initial area of 3.8cm², a potential change was monitored over 28 days for each
399 composite hydrogel cultured in proliferation or differentiation medium. Contraction of pure collagen
400 hydrogels was already observed from day 1 in proliferation medium (Figure 3A) and the surface area
401 progressively decreased with time to reach c.a 10% of its original value at day 28 (Figure 3B). The same
402 contraction amplitude was observed in differentiation medium (Figure 3C). This shrinkage was likely
403 due to BM-MSCs activities as the acellular hydrogels kept their original shape (Electronic Supporting
404 Information N°5). Composite and pure THA hydrogels did not shrink during the cell culture experiment.
405 On the opposite, a swelling phenomenon was observed proportionally to the THA content within
406 hydrogels. For pure THA hydrogels, the surface area increased during the time course of the
407 experiment to double at day 28. The presence of collagen within composite hydrogels showed a
408 tendency to inhibit the swelling as the area change was about 150% and 130% for the 1:5 and 1:2
409 ratios, respectively (Figure 3B and C). Again, the differentiation cell culture medium did not affect the
410 hydrogel surface area as similar results were obtained (Figure 3B and C).



411

412

413 **Figure 3:** Cell-laden collagen/THA hydrogel stability. (A) Macroscopic view of collagen/THA hydrogels
414 after one day (left) and 14 days (left) of cell culture. Changes in surface area of cellularized composite

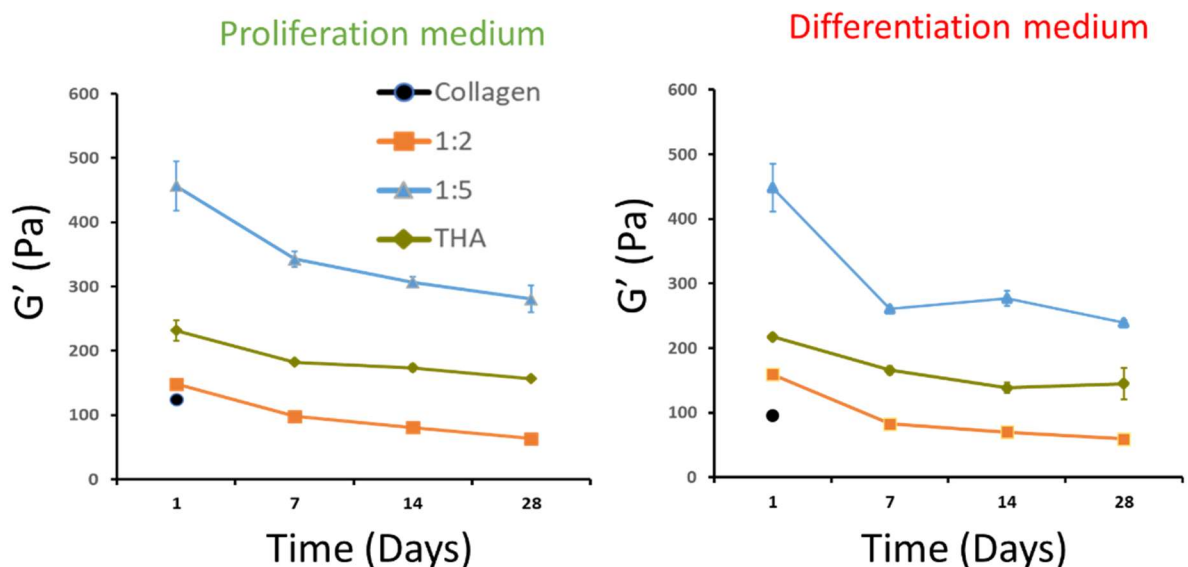
415 hydrogels cultured in proliferation (B) or differentiation medium (C) over 28 days. Results expressed
416 as a percentage of the initial area +/- SD (n=6).

417 3.3 Rheological properties of Collagen/THA composite hydrogels

418

419 The viscoelastic properties of composite hydrogels were investigated by rheometry. For pure collagen
420 hydrogels, a storage modulus, G' , around 100 Pa was measured at day 1. Unfortunately, the measure
421 was not possible from day 7 due to the extensive hydrogel contraction by cells, thereby reducing the
422 hydrogel surface and altering its shape. Regarding composite hydrogels, G' was slightly higher at day 1
423 (c.a 150 Pa) for the 1:2 ratio regardless of the culture medium used (Figure 4). For the 1:5 ratio, the
424 storage modulus measured at day 1 was around 450 Pa. The latter was much higher than that
425 measured for pure THA (around 200 Pa) at the same time point. Interestingly, the G' in these composite
426 hydrogels was higher than the sum of moduli measured in pure collagen and THA (Figure 4). It is worth
427 noticing that these composite hydrogels are composed of the same quantity of collagen and THA as
428 the pure hydrogels. The storage modulus of composite and pure THA hydrogels decreased during the
429 cell culture to loose around 30% of its original value. The decrease is mainly visible between day 1 and
430 7. The culture medium type did not seem to impact the hydrogel mechanical properties as no
431 significant differences were observed between the proliferation and differentiation medium (Figure
432 4).

433



434

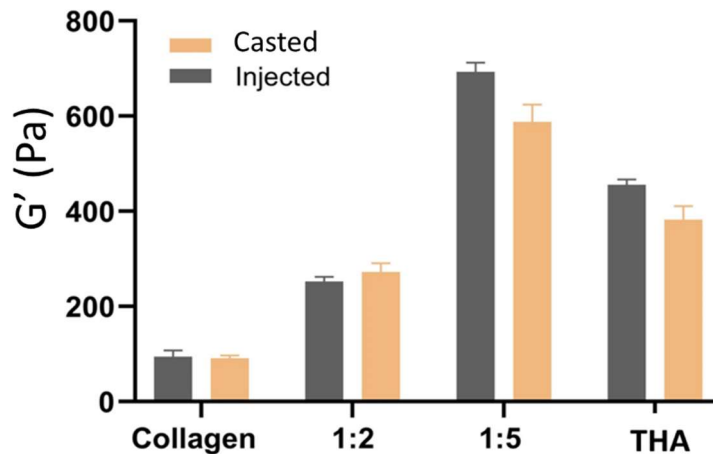
435 **Figure 4:** Evolution of the storage modulus of cell-laden collagen/THA composite hydrogels cultured
436 over 28 in proliferation or differentiation cell culture medium. Data presented as means \pm SD for 6
437 biological replicates.

438

439 3.4 Injectability of collagen/THA composite hydrogels

440

441 The different hydrogel formulations were manually injected through a 22G needle as seen in the video
442 (Video Supplementary information N°3). No significant differences were observed in storage moduli
443 between casted and injected hydrogels (Figure 5).



444

445 **Figure 5:** Mechanical properties of casted or injected collagen/THA hydrogels (n=6).

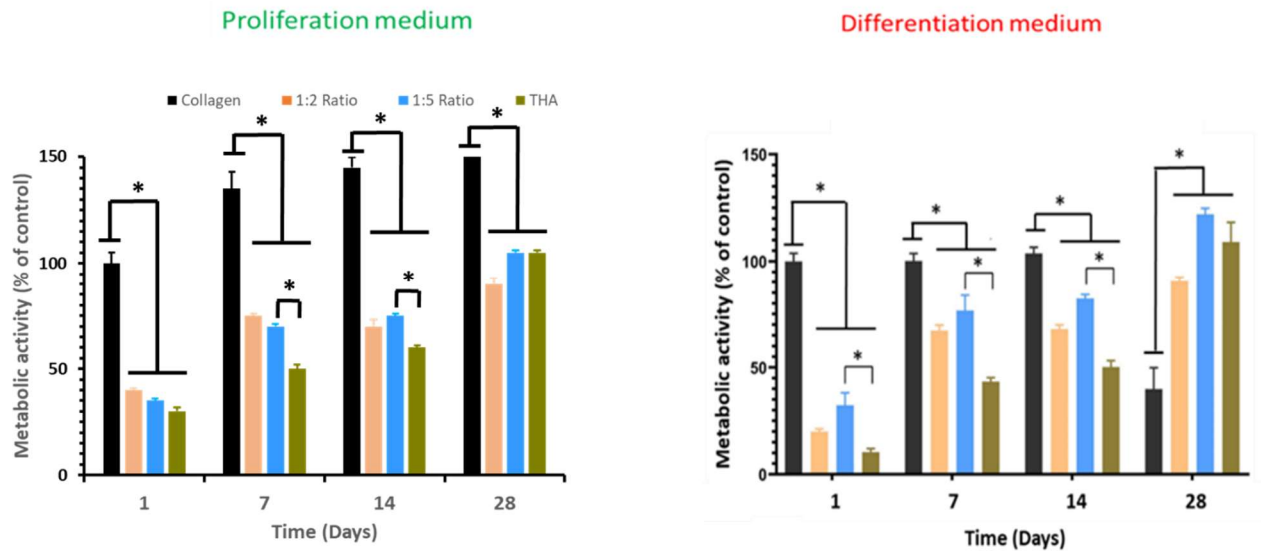
446

447 3.5 BM-MSC metabolic activity within collagen/THA composite hydrogels

448

449 Cell metabolic activity of BM-MSCs cultivated within pure collagen hydrogels with the proliferation
450 medium stayed quasi constant over the cell culture period (Figure 6). Metabolic activity of BM-MSCs
451 at day 1 was significantly lower in composite and pure THA hydrogels with approximately 30% of the
452 metabolic activity observed in pure collagen hydrogels. However, this value progressively increased to
453 reach around 100% at day 28 but remained lower than that in pure collagen hydrogel. At day 7 and 14,
454 the cell metabolic activity in collagen/THA hydrogels with the 1: 5 ratio was significantly higher than
455 that measured in pure THA hydrogels (Figure 6).

456 In the differentiation medium, the trend was similar. At day 1, the metabolic activity of BM-MSCs
457 within pure collagen hydrogels was at least the triple compared to the other hydrogel formulations.
458 This cell metabolic activity was constant until day 14 but decreased by 60% at day 28. In composite
459 and pure THA hydrogels, the cell metabolic activity increased to reach about 100% at day 28 (Figure
460 6). In addition, the value measured in composite hydrogels with the 1:5 ratio was higher than that
461 measured in pure THA hydrogels until day 14.



463

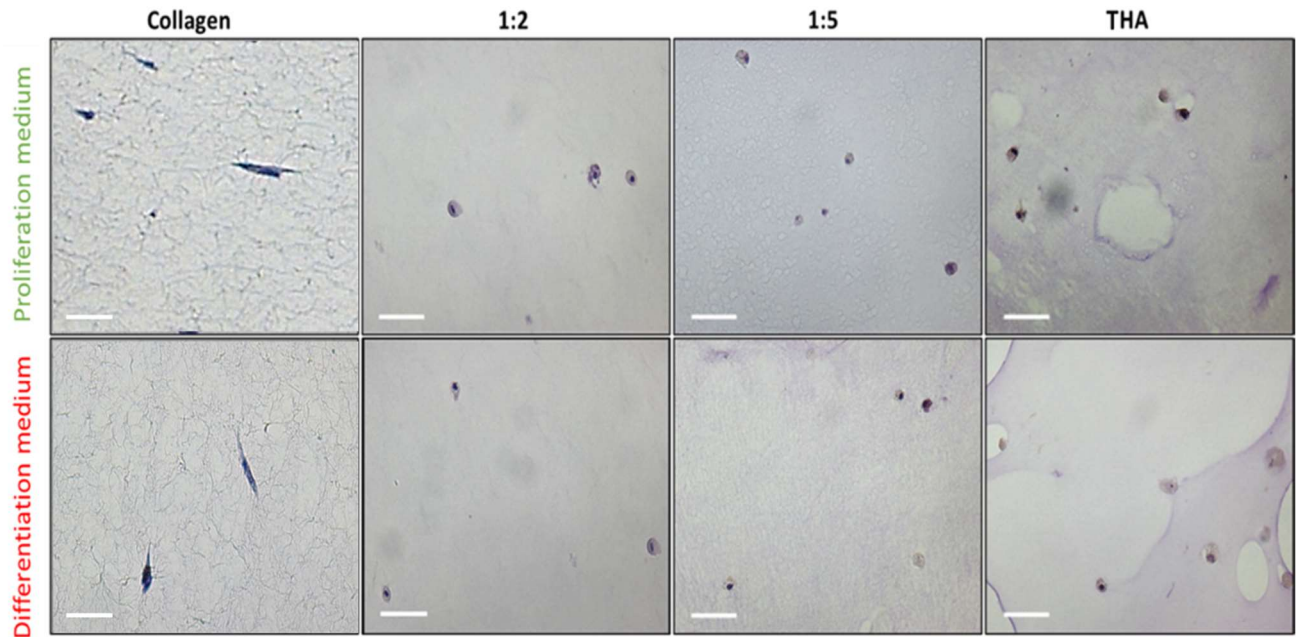
464 **Figure 6:** Metabolic activity of human BM-MSCs within collagen/THA hydrogels cultured in
 465 proliferation or differentiation medium over 28 days (n=4), *P<0.05 (Kruskal-Wallis test, post-hoc test:
 466 Dun).

467

468 3.6 Cell morphology

469

470 BM-MSCs cultivated within pure collagen hydrogels exhibited a dendritic elongated shape regardless
 471 of the culture medium used (Figure 7). When THA was added to form composite hydrogels, the cell
 472 morphology changed. Cells appeared rounded in 1:2, 1:5 and pure THA hydrogels. For the composite
 473 and pure THA hydrogels, the culture medium did not impact cell morphology.



474

475 **Figure 7:** Cell morphology of BM-MSCs cultured within collagen/THA composite hydrogels with
 476 proliferation or differentiation medium at day 14. Images obtained after Mayer's Hematoxylin staining.

477 Bar: 50 μ m

478

479 3.7 Gene expression of ECM proteins

480

481 The differentiation of BM-MSCs into NP-like cells was first investigated by their ability to express mRNA
 482 for specific ECM proteins such as collagen 2 and aggrecan. Aggrecan (ACAN) gene expression was very
 483 low in proliferation medium at day 1 and 7 regardless of the hydrogel type. At day 14, this gene
 484 expression increased in all hydrogels to reach c.a 7 times the gene expression measured for the
 485 calibrator point (Cells in pure collagen hydrogels culture in differentiation medium at day1) (Figure 8A).
 486 The ACAN gene expression did not increase at day 28 except for pure THA hydrogels which raised by
 487 10 times compared to day 14 (Figure 8A). Regarding hydrogels cultured in differentiation medium,
 488 ACAN gene expression was detected as early as day 7 with a 2-3 fold increase in the hydrogels
 489 containing THA. This expression was correlated to the THA content at this time point. The ACAN
 490 expression then slightly increased in collagen/THA composite hydrogels at day 28 and tripled in pure
 491 THA hydrogels. In comparison to hydrogels cultured in proliferation medium, ACAN gene expression
 492 at day 14 and 28 was not significantly different, regardless of the type of hydrogel studied.

493 BM-MSCs encapsulated within hydrogels weakly expressed Collagen 2 until day 14 when cultured in
 494 proliferation medium (Figure 8B). This gene expression level remained very low for pure collagen
 495 hydrogels whereas it increased in composite and pure THA hydrogels at day 28 (Figure 8). At this time

496 point, COL2A1 gene expression was c.a 10 times higher in collagen/THA composite hydrogels
497 compared to pure collagen. In addition, the gene expression in pure THA was 10 times higher than that
498 in composite hydrogels. Cells cultured in differentiation medium expressed COL2A1 gene already at
499 day 7 with an expression 100 times higher than that measured in control samples (Cells in Pure
500 Collagen at day 1). At this time point, COL2A1 gene expression was 10 times higher in composite and
501 pure THA hydrogels compared to the gene expression in pure collagen hydrogels. COL2A1 gene
502 expression increased until day 14 in all hydrogels except for the 1:2 ratio to be multiplied by 10 and
503 100 times for 1:5 composite and pure THA hydrogels, respectively. It is worth noting that COL2A1 gene
504 expression was around 100 times higher when BM-MSCs were cultivated in differentiation medium
505 compared to the culture in proliferation medium (Figure 8B).

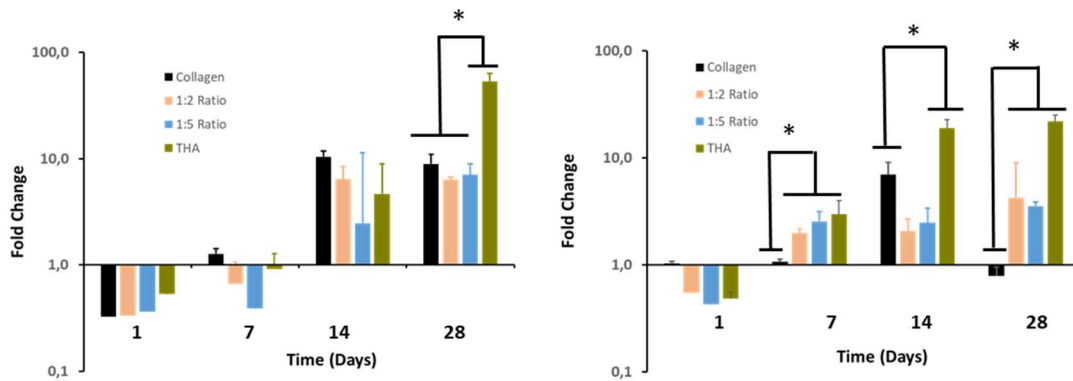
506 The collagen 1 (COL1A1) gene expression, an osteoblast and fibroblast marker, was very low in all
507 hydrogels and seemed to be inhibited by the THA content when cultured in proliferation medium
508 (Figure 8C). Compared to pure collagen hydrogels, COL1A1 gene expression in composite and pure THA
509 hydrogels was at least 5 times lower until day 14 (Figure 8C). The differentiation medium had a positive
510 impact on the COL1A1 gene expression in all hydrogels. However, the THA content seems to
511 downregulate the COL1A gene expression (Figure 8C). This expression decreased in all types of
512 hydrogels at day 28 to reach the level observed for BM-MSCs cultured in proliferation medium.

513

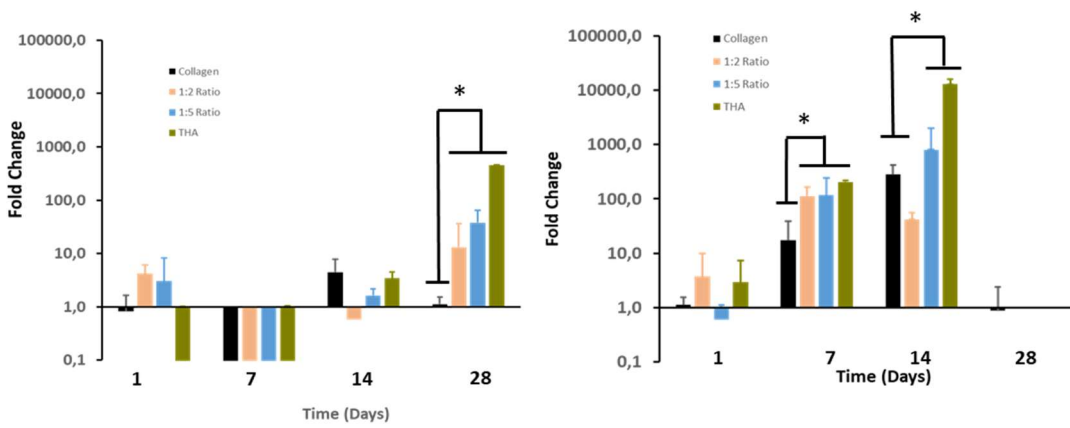
Proliferation medium

Differentiation medium

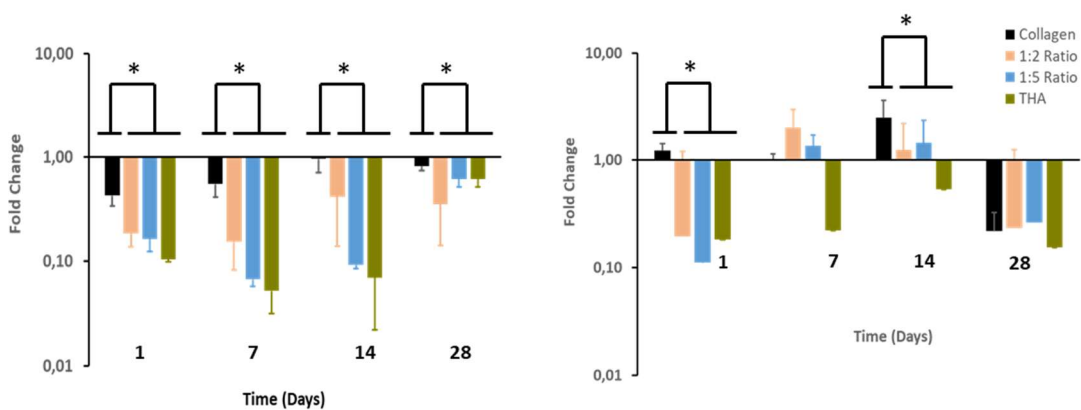
ACAN



COL2A1



COL1A1



514

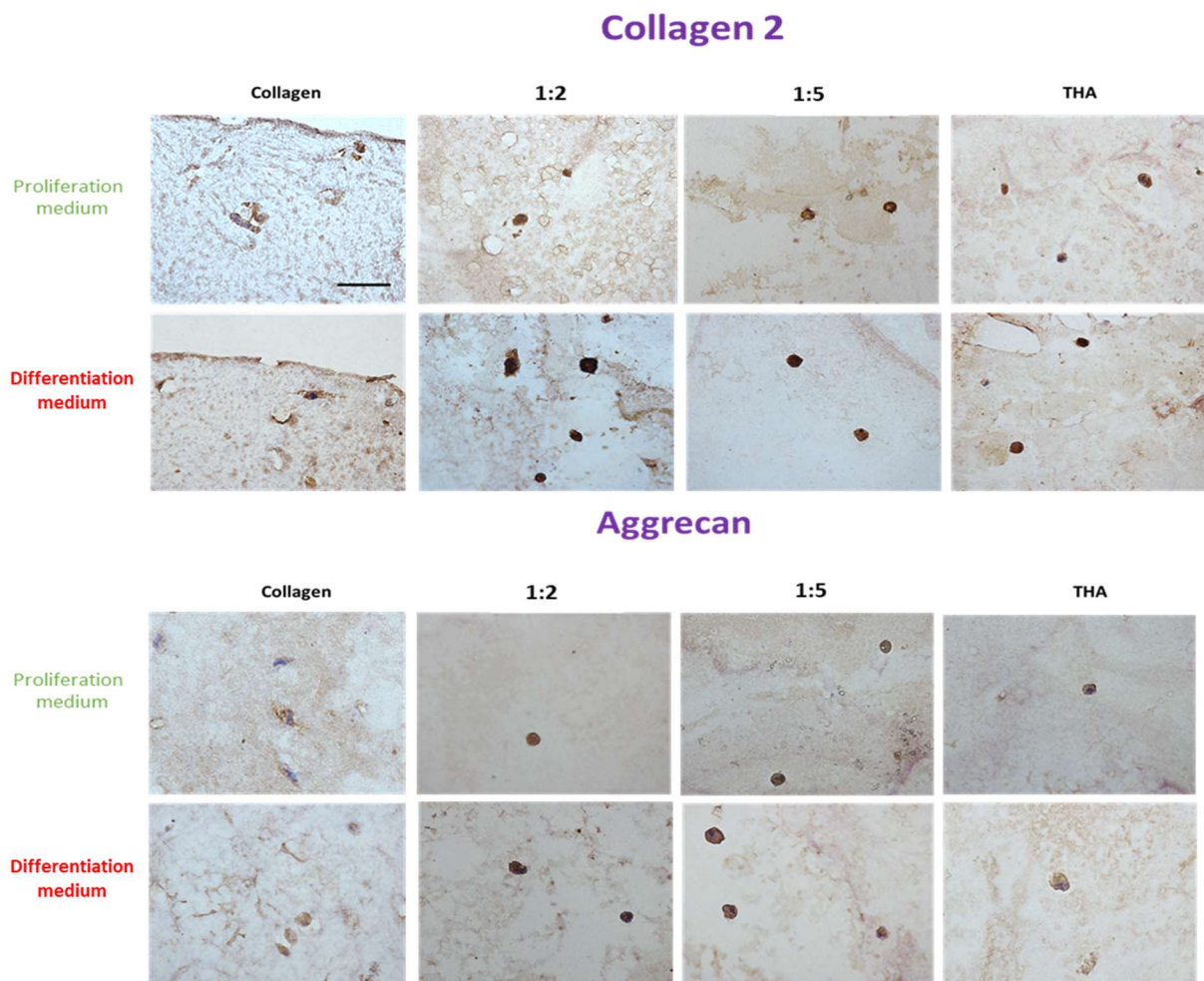
515 **Figure 8:** Gene expression of Aggrecan (ACAN), Collagen 2 (COL2A1) and Collagen 1 (COL1A1) of
 516 human BM-MSCs within composite hydrogels. (n=3). Fold change relative to the calibrator point (Gene
 517 expression within pure collagen hydrogels at day 1 in proliferation medium). *: P<0.05 (Kruskal-Wallis
 518 test, post-hoc test: Dun).

519 3.8 BM-MSC protein expression for NP constituents

520

521 BM-MSCs encapsulated in pure collagen hydrogels exhibited a weak collagen 2 labelling at day 14 when
522 they were cultured in proliferation medium (Figure 9). The THA content within composite and pure
523 THA hydrogels had a positive effect on collagen 2 production as the labelling appeared darker in these
524 conditions. When cultured in differentiation medium, the collagen 2 immunodetection was stronger
525 in all hydrogels (Figure 9). The labelling also had a higher intensity in cells encapsulated within
526 hydrogels containing THA (composite and pure THA) without any difference between the different
527 conditions.

528 The immunodetection of aggrecan was weak in BM-MSCs cultivated in pure collagen hydrogels at day
529 14 (Figure 9). In contrast, cells exhibited a strong labelling in composite and pure THA hydrogels. No
530 difference was visible between the different hydrogels which contain THA. Additionally, the type of
531 culture medium used did not seem to affect the production of aggrecan, as the images for both
532 mediums appeared similar (Figure 9).



533

534 **Figure 9:** Collagen 2 and aggrecan Protein expression of BM-MSCs cultured within collagen/THA
535 composite hydrogels at day 14.

536 3.9 BM-MSc gene expression of differentiation markers

537

538 SOX 9, a major chondrogenic and NP marker, was expressed in BM-MSCs cultivated in pure collagen
539 hydrogels in presence of proliferation medium. This expression was constant over 28 days. The
540 presence of THA within hydrogels led to a weak expression of SOX 9 by cells until day 14. At day 28,
541 SOX 9 gene expression increased in composite hydrogels and was proportional to the THA content
542 (Figure 10A). BM-MSCs cultivated in pure collagen hydrogel with the differentiation medium strongly
543 expressed SOX 9 at day 7 and 14, *i.e* around 6 times the SOX 9 expression at day 1. Afterwards, SOX 9
544 expression decreased and returned to its basal level at day 28. Cells cultured within composite and
545 pure THA hydrogels had a similar trend with relatively lower gene expression of SOX 9 compared to
546 pure collagen hydrogels, except for day 14.

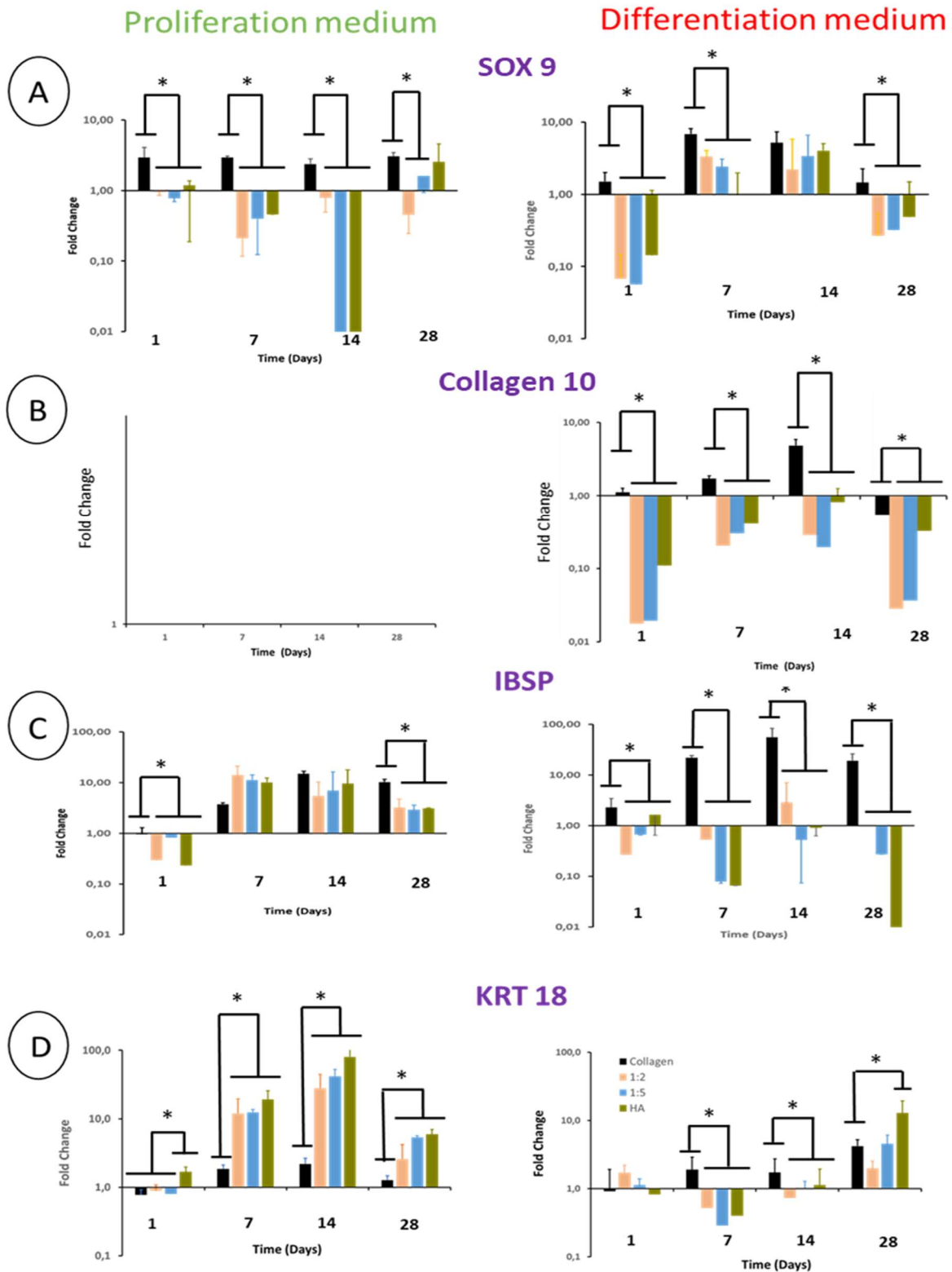
547 Regardless of the type of hydrogel analyzed, BM-MSCs cultured in proliferation medium did not
548 express COL10 (collagen 10), which is the main marker of hypertrophic chondrocytes. (Figure 10B).
549 Cells in pure collagen hydrogels, however highly expressed Collagen 10 during their culture in
550 differentiation medium (Figure 10B). This expression increased until day 14 to reach 5 times its basal
551 value. Interestingly, the presence of THA within hydrogels decreased the COL10 gene expression at
552 least by 10 (Figure 10B).

553 Integrin-binding sialoprotein (IBSP) is an osteoblast marker but also a positive marker of mature
554 chondrocytes. It is considered to be a negative marker of NP cell differentiation. IBSP gene expression
555 increased in BM-MSCs cultivated in proliferation medium until day 7 (Figure 10C). Compared to pure
556 collagen, this gene expression was slightly higher in composite and pure THA hydrogels at day 7, similar
557 at day 14 and lower at day 28. In differentiation medium, IBSP expression measured in pure collagen
558 hydrogels increased to reach 20 times its basal level at day 14. In contrast, this gene expression was
559 low in composite and pure THA hydrogels during the cell culture (10-100 times less) (Figure 10C).
560 Compared to the proliferation medium, IBSP was significantly less expressed in differentiation medium
561 for the composite and THA hydrogels but not for pure collagen hydrogels.

562 Cytokeratin 18 (KRT18) is a specific marker of healthy NP cells. Its gene expression increased until day
563 14 (Figure 10D) for BM-MSCs cultivated in proliferation medium. THA had a positive effect on KRT18
564 expression as the levels in composite and pure THA hydrogels were at least 5 times higher than those
565 observed in pure collagen hydrogels (Figure 10D). KRT 18 gene expression in differentiation medium
566 were much lower compared to cells cultivated in proliferation medium until day 14 and were similar
567 at day 28. At this time point, the THA content seem to have a positive impact on KRT 18 expression.

568

569



570

571 **Figure 10:** Gene expression of SOX 9, collagen 10 (COL10), integrin binding sialoprotein (IBSP) and
 572 cytoke­ratin 18 (KRT18) within collagen/THA composite hydrogels. (n=3). Fold change relative to the
 573 calibrator point (Gene expression within pure collagen hydrogels at day 1 in proliferation medium). *:
 574 P<0.05 (Kruskal-Wallis test, post-hoc test: Dun).

575 4. Discussion

576 In this study, we analyzed the impact of physical and biochemical properties of injectable collagen/THA
577 composite hydrogels on the differentiation of human BM-MSCs into NP-like cells. To achieve this, we
578 modulated the THA content in the hydrogels, tailoring the mechanical properties, hydration,
579 biochemical cues and the fibrillar collagen structure to closely resemble the Nucleus Pulposus.
580 Additionally, we evaluated the potential of pro discogenic growth factors, namely TGF β 1 and GDF5, to
581 potentialize the effect of biomimetic collagen/THA hydrogels ^{32,33}. **These growth factors were used in
582 combination to avoid the chondrocyte differentiation triggered by TGF β 1 when used on its own and
583 added in the culture medium at a concentration known to differentiate stem cells into NP-like cells in
584 2D culture ³².**

585 Synthesizing collagen/THA composite hydrogels is challenging as these biopolymers strongly interact
586 in solution to form polyionic complexes (PICs) which precipitate ²⁴. To prevent the PICS formation and
587 obtain a homogenous hydrogel, hyaluronan and collagen are usually used in neutral solutions and
588 crosslinked to form a hybrid network ³⁴. Under these conditions, fibrillogenesis is inhibited. In the
589 present study, our strategy was to simultaneously gel collagen and THA by pH increase and the
590 utilization of HRP and H₂O₂. The gelling kinetic of both polymers was tuned to allow for collagen
591 fibrillogenesis before THA crosslinking without PICs formation ²⁶. The presence of fibrils is crucial to
592 mimic the native form of NP tissue and its specific topography ³⁵. Moreover, it has been shown that
593 the collagen topography in the form of fibers promoted the MSCs proliferation and differentiation ³⁶.
594 With the aim of promoting NP regeneration, hydrogels have to be injectable and stable enough in situ
595 to act as a temporary scaffold for cells. Regardless of their composition, pure and composite hydrogels
596 developed in the present study were injectable and their mechanical properties were not altered by
597 the injection. Nevertheless, these hydrogels did not exhibit the same stability after gelling. The BM-
598 MSC encapsulation within pure collagen hydrogels led to an extensive contraction of the collagen
599 network as described by other groups ³⁷. Hence, cell-laden collagen hydrogels do not seem to be
600 promising biomaterials for NP regeneration. On the opposite, pure THA and collagen/THA composite
601 hydrogels swelled during the cell culture experiment. This swelling suggests that MSCs remodeled their
602 environment, possibly by breaking some chains or crosslinking points ³⁸. In collagen/THA composite
603 hydrogels, collagen stabilizes the structure because the swelling is smaller for 1:2 than to 1:5 hydrogels.
604 Hence, the combination of collagen with THA seems to be the best compromise for the long-term
605 stability of the scaffold. As previously seen, the degree of hydration is higher than 80% for composite
606 and pure THA hydrogels ²⁶. The observed swelling is correlated to a decrease of mechanical properties
607 at day 7. This is logical as the stiffness depends on the number of crosslinking points. For pure collagen,
608 contraction stiffened hydrogels, leading to a decreased O₂ and nutrients diffusion ^{39, 40} As THA and

609 composite hydrogels did not shrink, diffusion properties are not altered. The absence of contraction
610 can be explained by the original stiffness of composite and pure THA hydrogels which is at least twice
611 as high in composite hydrogels than that in pure collagen hydrogels^{39,40}. It could also be explained by
612 the biochemical nature of composite and pure THA hydrogels. Contraction depends on the cell/matrix
613 interactions via cellular receptors. Indeed, cells need to spread and organize their cytoskeleton to
614 contract the polymeric network⁴¹. When THA is added in large quantity within hydrogels, the
615 cell/matrix interactions via CD 44 and RHAMM receptors are weak and prevent contraction²¹. This is
616 confirmed by the observation of a rounded morphology in pure THA or collagen/THA composite
617 hydrogels, suggesting this weak interaction. The cell morphology within composite hydrogels
618 resembles the NP cell one^{42, 43}. This was expected as natural NP cells are in a highly hydrated
619 environment consisting of a large quantity of polysaccharides⁴.

620 THA also had an impact on cell viability. Cell adhesion was optimal for pure collagen hydrogels but
621 decreased when the THA content raised in composite hydrogels. This shows that collagen is required
622 to get the optimal cell viability as it provides the adequate cues to cells for adhesion and survival^{23,26}.
623 Cell viability in pure collagen hydrogels was constant suggesting an inhibition of cell multiplication due
624 to the hydrogel contraction^{31,44}. In differentiation medium, a drop of cell viability was observed at Day
625 28. This could be due to an apoptotic phenomenon as previously seen for fibroblasts cultured in
626 contracted collagen hydrogels⁴⁵. On the opposite, hydrogel stability and adequate biochemical cues
627 seem to promote BM-MSC viability. Collagen/THA composite hydrogels with the 1:5 and 1:2 ratio have
628 the same collagen content than pure collagen hydrogels but they did not contract thanks to the THA
629 network. This allows rapid medium diffusion and cell proliferation as the porosity is great. Despite a
630 weak interaction with their matrix, BM-MSCs cells proliferated within pure THA hydrogels, suggesting
631 a remodeling of hydrogel to increase adherence. Noteworthy, the rheological properties of composite
632 hydrogels did not seem to impact cell viability, as BM-MSCs had the same metabolic activity in the 1:2
633 and 1:5 hydrogels despite a different stiffness.

634 The performance of a cellularized biomaterial to promote the Nucleus Pulposus regeneration is
635 evaluated by the cellular production of aggrecan and collagen 2 which restore the NP hydration and
636 its native fibrillar network. In contrast, the expression of collagen 1 has to be low as it is a marker of
637 Annulus Fibrosus cells and osteoblast differentiation^{46, 47}. TGF β 1 is known to promote BM-MSC
638 differentiation into chondrocytes⁴⁸ whereas GDF5 favors the NP cell phenotype appearance³². In
639 addition, these growth factors act in synergy to enhance the production of NP extracellular matrix and
640 NP specific markers³². The THA content had a slight effect on the gene expression of aggrecan within
641 composite hydrogels. Besides, the differentiation medium seems to accelerate the expression of this
642 proteoglycan without increasing its level. As aggrecan gene expression is higher in pure THA hydrogels
643 than in composite or pure collagen hydrogels, a weak adhesion seems to be required to promote this

644 gene expression. Collagen 2 gene expression is only detected at day 28 when cells are cultured in
645 proliferation medium and the THA content has a strong impact on this gene expression. BM-MSCs in
646 pure collagen hydrogels do not express collagen 2. When TGF β 1 and GDF5 were added, the collagen 2
647 expression was detected from day 7 and was 10 times higher. TGF β 1 is known to trigger the collagen
648 2 gene expression ^{49, 50}. In addition, a high THA content is also required for an optimal collagen 2
649 production. This was confirmed on histological section after collagen 2 labelling. The collagen 1 gene
650 expression was very low within all types of hydrogels, thereby evidencing the absence of
651 differentiation into osteoblasts and fibroblasts. Despite a higher gene expression of COL1A1 in the
652 differentiating medium, the ratio collagen 2/collagen 1 remained very high indicating the absence of
653 fibroblast differentiation. Last, the presence of THA seems to inhibit the expression of collagen 1 as
654 this expression is lower in composites and pure THA hydrogels. Hence, the combination of TGF β 1 and
655 GDF 5 with a high THA content, *i.e* a high degree of hydration and weak cell/matrix interaction, seems
656 to act in synergy to differentiate BM-MSCs into a NP like phenotype.

657 SOX 9 is a major marker of NP cells but is also expressed by chondrocytes ⁵¹⁻⁵³. SOX 9 is only expressed
658 at day 28 in proliferation medium, suggesting a weak differentiation. The addition of GDF5 and TGF β 1
659 triggers the cell differentiation toward a chondrocyte-like phenotype in composite hydrogels but was
660 higher in pure collagen hydrogels. It has been shown that the matrix stiffness is a major stimulus to
661 trigger chondrocyte differentiation ⁵⁴. As pure collagen hydrogels contract, the stiffness increased
662 during the cell culture to reach around tens of kPa⁵⁵. So, BM-MSCs are in the appropriate environment
663 to differentiate into chondrocytes. The SOX 9 gene expression in pure collagen was associated to a
664 high gene expression of collagen 10 when cells were cultured with GDF5 and TGF β 1. Hence, cells
665 differentiated into hypertrophic chondrocytes probably due to TGF β 1 ^{56 57}. This could also explain the
666 decrease in metabolic activity observed at day 28 as hypertrophic chondrocytes are prone to
667 senescence. In addition, hypertrophic chondrocytes mineralized their matrix and eventually die ^{58, 59}.
668 Hence, pure collagen hydrogels associated with GDF5 and TGF β 1 are not adequate biomaterials to
669 differentiate BM-MSCs into NP-like cells. It is worth noticing that the collagen 10 expression was very
670 low in composites and pure THA hydrogels, thereby evidencing the protective effect of THA. This could
671 be due to the stability and the lower mechanical properties of these hydrogels compared to pure
672 collagen ones. It has been shown that mechanical properties around 1kPa (storage modulus, G') were
673 more suitable to trigger differentiation into NP cells. Higher mechanical properties usually lead to
674 osteogenic differentiation ⁶⁰. The hypertrophic phenotype for pure collagen hydrogels did not appear
675 when cells were cultured in proliferation medium. Combined with a low SOX 9 expression, this
676 confirms the weak differentiation in the proliferation medium.

677 To discriminate the NP cell from the chondrocyte phenotype, IBSP and KRT 18 are relevant markers ⁵³,
678 ⁶¹. IBSP is a chondrocyte and osteoblast marker weakly expressed in NP cells, and it is thereby regarded

679 as a negative marker for NP cells⁶¹. In contrast, KRT18 is a marker highly expressed by NP cells and
680 weakly expressed by chondrocytes⁶¹. Surprisingly, IBSP was highly expressed by cells in proliferation
681 medium. It has been recently shown that IBSP is an aging marker of multipotent stem cells⁶². Hence,
682 without gene expression of SOX 9, we can hypothesize that BM-MSCs are not differentiated until day
683 14 in the proliferation medium. At day 28, IBSP gene expression decreased in composite hydrogels but
684 KRT18 was high. It suggests that the NP cell differentiation starts at day 28 in proliferation medium.
685 When GDF5 and TGF β 1 was used, IBSP expression was very low in composite and pure THA hydrogels
686 but high in pure collagen hydrogels. This shows that collagen hydrogels with a high stiffness promote
687 the hypertrophic chondrocyte phenotype whereas a high THA content within composite hydrogels
688 promote the NP cell phenotype. This is confirmed at day 28 when the KRT18 gene expression became
689 high.

690 Biomaterials used as cell carrier for NP regeneration have to promote stroma cell differentiation and
691 provide appropriate physical properties to restore the NP function. Then, they have to be remodeled
692 into a neo tissue by cells. Hydration is the most important physical property required to treat NP
693 degeneration as it is responsible for the restoration of disc height. For this reason, highly hydrated
694 hydrogels were used in the first place. Synthetic polymers such as PEG or pNIPAM are interesting as
695 they are easy to synthesize and their physical properties can be tuned to mimic the NP ones^{15, 16}. In
696 addition, some of them are biologically active and promote BM-MSCs differentiation into NP cells¹⁵.
697 However, they are not degraded by cells⁶³. Therefore, their remodeling is not controllable. Natural
698 biopolymers from marine organisms such as agarose or alginate have the same drawbacks¹⁶. That's
699 the reason why collagen and Hyaluronic acid were chosen to synthesize biomimetic hydrogels in this
700 study because they are degradable and allow for biomaterial remodeling. The major drawback of
701 collagen/HA hydrogels is their poor mechanical properties despite a higher performance compared to
702 fibrin hydrogels⁶⁴. To reach the NP physical properties, they have to be cross-linked⁴. Cross-linkers
703 such as EDC or glutaraldehyde can be harmful and decrease the hydrogel degradation^{4, 34}. In addition,
704 mixing collagen and HA with cross-linkers leads to materials without collagen fibrils. Hence, the NP
705 topography is lost. Using enzymatic cross-linking via HRP, cell survival was favored in biomimetic
706 collagen/THA hydrogels due to the mild conditions²⁶. Hence, we restored the topographical feature of
707 NP despite collagen II is not used. Usually, research performed on Collagen/HA hydrogels focuses on
708 an unique formulation and often uses a low HA content to prevent formation of polyionic complexes
709³⁴. The appropriate quantity of both biopolymers to reach a high degree of hydration and cell
710 differentiation is not studied. Here, we performed a systematic study to find that a high content of
711 THA and the addition of discogenic factors are required to promote such differentiation. Besides,
712 collagen is essential to obtain a high cell viability. In addition, the enzymatic crosslinking allows for
713 remodeling by cellular enzymes as previously shown²⁶. We found the compromise between hydration,

714 cell adhesion, mechanical properties and the ability to promote cell differentiation to generate a
715 biomaterial suitable for NP regeneration.

716

717

718 **5. Conclusions**

719

720 This study shows that the combination of a high THA content with discogenic growth factors is required
721 to promote the differentiation of BM-MSCs into NP-like cells within collagen/THA composite
722 hydrogels. Hydrogel stability and mechanical properties around 500 Pa are strong stimuli for long term
723 cell viability whereas collagen ensures cell adhesion and short-term survival. A high THA impacts
724 cell/matrix interactions and increase the degree of hydration to direct the cell morphology and
725 phenotype toward NP cell phenotype. Using a 1:5 collagen/THA ratio, similar to that in NP, cells adopt
726 the characteristic rounded morphology and highly expressed the specific NP extracellular matrix
727 biopolymers *i.e* aggrecan and collagen 2. In contrast, pure collagen hydrogels are not adequate for NP
728 regeneration as cells differentiated into hypertrophic chondrocytes. Hence, injectable and biomimetic
729 hydrogels with a high degree of hydration (owing to a high THA content,) with a small proportion of
730 fibrillar collagen seems to be promising biomaterials for NP regeneration.

731

732

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737 advices.

738 **Supporting Information**

739 Supporting Information is available from XX.

740 **Conflicts of interest**

741 There are no conflicts of interest to declare

742

743

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