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1 **Culture temperature affects redifferentiation and cartilaginous extracellular matrix**
2 **formation in dedifferentiated human chondrocytes**
3

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17

18 **Running title:** Culture temperature in chondrocytes
19

20 **Abstract**

21 To date, there have been few studies on how temperature affects the phenotype and
22 metabolism of human chondrocytes. Thus, the purpose of this study was to elucidate the
23 effects of culture temperature on chondrocyte redifferentiation and extracellular matrix
24 (ECM) formation using dedifferentiated mature human chondrocytes *in vitro*.
25 Dedifferentiated chondrocytes were cultured in a pellet culture system for up to 21 days. The
26 pellets were randomly divided into three groups with different culture temperature (32°C,
27 37°C, and 41°C). Chondrocyte redifferentiation and ECM formation were evaluated by wet
28 weight, messenger ribonucleic acid (mRNA), histological, and biochemical analyses. The
29 results showed that the wet weight and the mRNA expressions of collagen type II A1 and
30 cartilage oligomeric matrix protein at 37°C were higher than the corresponding values at
31 32°C. The histological and biochemical analyses revealed that the syntheses of type II
32 collagen and proteoglycan were promoted at 37°C compared to those at 32°C, whereas they
33 were considerably inhibited at 41°C. In conclusion, the results obtained herein indicated that
34 temperature affects chondrocyte redifferentiation and ECM formation, and modulation of
35 temperature might thus represent an advantageous means to regulate the phenotype and
36 biosynthetic activity of chondrocytes.

37

38 **Keywords:** chondrocyte; temperature; extracellular matrix; differentiation; pellet culture.

39

Introduction

Articular cartilage (AC) is a hyaline cartilage composed of a dense cartilaginous extracellular matrix (ECM) with sparse distribution of highly specialized cells called chondrocytes. Recently, tissue engineering and cell-based therapies have been explored for AC regeneration,[1] since AC displays a limited capacity for renewal and self-repair.[2] Autologous chondrocyte implantation (ACI) is a promising cell-based therapy for repairing AC defects.[3] However, ACI poses several challenges. Harvested chondrocytes must be expanded to obtain a large number of cells for transplantation, and yet, this process results in the induction of chondrogenic phenotype loss (i.e., dedifferentiation),[4,5] causing fibro-cartilage-like remodeling. Thus, characterization of the factors regulating the chondrogenic phenotype is desired for inducing redifferentiation and hyaline cartilage for ECM formation. Candidate factors include the microenvironment, such as the presence of growth factors,[6] scaffolds,[7] and oxygen tension,[8] as well as mechanical stimuli.[9] While these factors have all been well studied, there are conversely few studies that have focused on the role of temperature in chondrocyte redifferentiation.[10] Environmental temperature is known to influence some tissues such as the skin. Interestingly, the temperature within the human knee joint is also influenced by the environmental temperature, with a mean temperature of approximately 32°C, which is 4–5°C lower than the inner body temperature.[11,12] However, most *in vitro* studies on chondrocytes or AC have been performed using a culture temperature of 37°C, which may not accurately reflect the *in vivo* temperature. In addition, the effect of a high-temperature environment, such as 41°C, remains unclear, although an intermittent heat stimulus (41°C) has been reported to potentially have a positive effect on ECM formation.[13,14]

To date, there are few studies on how temperature affects the chondrocyte phenotype and metabolism of mature human chondrocytes. We hypothesized that the culture temperature

64 may influence the ability of dedifferentiated chondrocytes to redifferentiate and produce
65 hyaline-like ECM. Therefore, the purpose of this study was to elucidate the effects of culture
66 temperature (from physiological- to high-temperature) on redifferentiation and ECM
67 formation using dedifferentiated mature human chondrocytes *in vitro*.

68

69 **Methods**

70 **Chondrocyte isolation and pellet culture**

71 Human chondrocytes were obtained from the femoral heads (International Cartilage
72 Repair Society grade 0) extracted during bipolar hip arthroplasty performed in two 62- and
73 89-year-old women (donors A and B, respectively), as previously described.[15] The Ethics
74 Committee of the Faculty of Medicine at Kyoto University approved the procedure, and
75 informed consent was obtained from the donors. The isolated cells from the donors were
76 separately expanded in Dulbecco's modified Eagle medium/Ham's F12 (Nacalai Tesque Inc.,
77 Kyoto, Japan) containing 10% fetal bovine serum (Hyclone, Logan, UT), 50 U/mL penicillin
78 (Nacalai Tesque Inc.), and 50 μg/mL streptomycin (Nacalai Tesque Inc.) to dedifferentiate in
79 tissue culture dishes in a CO_2 incubator (5% CO_2 at 37°C with 95% humidity) until passage
80 two or three.

81 To provide a 3-dimensional environment, a pellet culture system was used.[16] The
82 expanded chondrocytes were trypsinized and subsequently resuspended in a chondrogenic
83 medium (Chondrogenic Differentiation Media BulletKit; Lonza, Walkersville, MD), which
84 was supplemented with 10 ng/mL recombinant human transforming growth factor-beta 3
85 (R&D Systems, Inc., Minneapolis, MN). Aliquots of 2.5×10^5 cells in 500 μL of the
86 chondrogenic medium were centrifuged at $250 \times g$ for 5 min in 15-mL polypropylene conical
87 tubes. The pelleted cells were randomly divided into three groups with different incubation

88 temperatures (32°C, 37°C, and 41°C). These temperatures were defined as follows: 32°C,
89 physiological intra-articular temperature; 37°C, conventionally used inner body temperature;
90 and 41°C, the threshold temperature for mammalian cell survival.[17,18] The pellets from donor
91 A were harvested at 3, 7, 14, and 21 days for analysis. The pellets from donor B cultured at
92 32°C and 37°C were harvested at 3 and 21 days to confirm the reproducibility of the findings
93 from donor A. Throughout the study, "*n*" indicates the technical replicates of the pellet
94 cultures.
95

96 **Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)**

97 The generated pellets (n = 3 pellets/group) were harvested after 3 and 7 days. The total
98 RNA was extracted and qRT-PCR was performed as previously described.[15] The target genes
99 and reference genes used were as follows: chondrogenesis markers (collagen type II A1
100 [*COL2A1*], aggrecan [*ACAN*], and cartilage oligomeric matrix protein [*COMP*]); a
101 fibro-cartilage maker (collagen type I A1 [*COL1A1*]); and reference genes (ribosomal protein
102 L13a [*RPL13a*] and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation
103 protein [*YWHAZ*]). Their specific primers are listed in Supplementary Table 1.

104 The data obtained by qRT-PCR were analyzed using the comparative threshold cycle
105 method. Briefly, the quantity of the target genes was normalized to the expression levels of
106 *RPL13a* and *YWHAZ*, which have been proven to be stable under different thermal
107 conditions.[19] The value of the calibration sample (cells cultured at 32°C on day 3) was set to
108 1, and the values for the other conditions were reported relative to that of the calibration
109 sample.
110

111 **Histological and immunohistochemical (IHC) analyses**

112 The generated pellets cultured in the three different temperatures obtained on days 7, 14,
113 and 21 were stained with safranin-O/fast green for assessment of proteoglycan deposition,
114 and with picrosirius red to visualize the collagen orientation and integrity under a polarizing
115 microscope (Eclipse 80i; Nikon, Tokyo, Japan).[20] IHC staining of type II and I collagen was
116 performed to detect each type of collagen deposition according to previously described
117 methods.[21] To semi-quantify the immunoreactivity of type II collagen at 32°C and 37°C on
118 day 21, the images from each group (n = 8 pellets/group) were measured using the ImageJ
119 program (National Institutes of Health, Bethesda, MD) as previously described.[14]

120

121 **Scanning electron microscope (SEM) observation**

122 SEM observation was performed to assess the ultra-microstructure of collagen fibers
123 using a SEM system (H-7650; Hitachi, Tokyo, Japan). The generated pellets on day 21 were
124 fixed and cut into two pieces. The specimen was mounted cut surface up on aluminum stubs.
125 The collagen fibers on the surface, superficial, sub-superficial, transition, and deep regions on
126 the cut surface of the pellets were observed.

127

128 **Measurement of glycosaminoglycan (GAG) and deoxyribonucleic acid (DNA) content**

129 The total GAG content in the pellets on days 14 and 21 (n = 6 pellets/group) was
130 measured using the1,9-dimethylmethylen blue colorimetric method.[22] Moreover, the DNA
131 content in these samples (n = 6 pellets/group) was assessed using the Quant-iT™
132 PicoGreen® assay (Invitrogen Ltd., Paisley, UK) following the manufacturer's instructions.
133 To estimate the GAG productive ability per cell, the GAG/DNA ratio was calculated (GAG
134 content/pellet ÷ DNA content/pellet).

135

136 **Statistical analysis**

137 JMP 11 software (SAS Institute, Cary, NC) was used for the statistical analyses.
138 Descriptive statistics were calculated as means and 95% confidence intervals. Statistical
139 significance for the donor A experiments was determined using the paired *t*-test for the
140 semi-quantitative evaluation of type II collagen, or by one-way analysis of variance using the
141 post-hoc multiple comparison Tukey-Kramer test for other experiments. For donor B,
142 statistical significance was determined using the paired *t*-test. The correlation between GAG
143 content and wet weight was examined using Pearson's correlation coefficient. The differences
144 observed were considered to be significant if the P value was < 0.05.

145

146 **Results**

147 The results from donors A and B showed similar trends. Therefore, only the results from
148 donor A are described below, whereas the results from donor B, which were used to confirm
149 the reproducibility of the findings from donor A, are described in Supplementary Fig. 1.

150

151 **Wet weight measuring**

152 Representative pellets generated at the three different temperatures are shown in Fig. 1(a).
153 The pellets generated at 32°C and 37°C showed a ball-like shape, whereas that at 41°C
154 showed a disc-like shape. Fig. 1(b) shows the wet weight changes over time. Although the
155 wet weight was heavier at 32°C than at the other temperatures on day 3, the heaviest pellets
156 were those cultured in 37°C obtained on days 14 and 21. On the other hand, at 41°C, the wet
157 weight was significantly lighter than at the other temperature, and did not change over time.

158

159 **Gene expression analysis**

160 Gene expression analysis related to the cartilaginous ECM was performed on days 3 and
161 7 to analyze the early effects of temperature (Fig. 2). The gene expressions analyzed were
162 found to be significantly down-regulated at 41°C. Conversely, the expressions of *COL2A1*,
163 *COL1A1*, *ACAN*, and *COMP* were all up-regulated on day 7 compared to those on day 3 at
164 32°C and 37°C. The expressions of *COL2A1* and *COMP* were significantly more
165 up-regulated at 37°C than at 32°C on days 3 and 7, whereas no significant differences were
166 observed for the expressions of *COL1A1* and *ACAN* in this early phase.
167
168 **Histological and IHC analyses**
169 Representative images are shown in Fig. 3. Safranin-O staining, type II collagen IHC
170 staining, and picrosirius red staining revealed a progressive deposition at 32°C and 37°C, but
171 not at 41°C. IHC staining of type I collagen showed early and intense deposition at 32°C and
172 37°C, but not at 41°C. Picrosirius red staining at 32°C was observed not only in the
173 superficial region but also in the deep region (Fig. 3 [white arrow]) on days 14 and 21, while
174 that at 37°C was observed mainly in the superficial region. To clarify the differences in type
175 II collagen deposition between 32°C and 37°C on day 21, semi-quantitative evaluation was
176 performed. The mean intensity per pixel (Fig. 4a) and mean percentage of type II collagen
177 positive area (Fig. 4b) were found to be significantly higher at 37°C than at 32°C.
178
179 **SEM observation**
180 Fig. 5 shows representative images of the surfaces and cut surfaces of the pellets on day
181 21. While the surface of the pellets at 32°C and 37°C appeared even and smooth, these
182 characteristics were not observed at 41°C. In the superficial region, dense and layered
183 collagen fiber formations parallel to the surface were observed at 32°C and 37°C, whereas no

184 collagen formations were observed at 41°C. In the sub-superficial region, the collagen fibers
185 were randomly oriented, and they appeared sparse through the deep region. The collagen
186 fibers in the transition and deep regions at 32°C appeared denser than at 37°C.
187

188 **Measurement of GAG and DNA content**

189 The GAG content per pellet at 41°C was significantly lower than that at the other
190 temperature environments (Fig. 6a). The GAG content in the pellet generated at 37°C tended
191 to be higher than that obtained at 32°C. Moreover, the DNA content per pellet at 41°C was
192 significantly lower than that at the other temperature environments (Fig. 6b). When the
193 GAG content was normalized according to the DNA content, the value was found to be
194 significantly higher at 37°C compared to at 32°C on day 21 (Fig. 6c). The GAG content and
195 the wet weight had a strong positive correlation ($R^2 = 0.91$, $P < 0.01$, $n = 36$)
196 (Supplementary Fig. 2).
197

198 ## Discussion

199 Temperature, which can be manipulated easily in the cell culture process, and possibly
200 also in the clinical treatment, may be one of the key microenvironmental parameters
201 regulating the chondrogenic phenotype and ECM formation. We investigated the effects of
202 three different culture temperatures (32°C, 37°C, and 41°C) on the ability of dedifferentiated
203 mature human chondrocytes to redifferentiate and form ECM *in vitro*. To the best of our
204 knowledge, this is the first report on the effect *in vivo* intra-articular temperature on human
205 chondrocyte metabolism *in vitro*. Our results demonstrated that the wet weight measured up
206 to day 21 showed time-dependent increases at 32°C and 37°C, suggesting ECM accumulation
207 (Fig. 1b). However, on the other hand, the wet weight at 41°C did not change over time.

208 Moreover, the ECM-related genes (*COL2A1, COL1A1, ACAN*, and *COMP*) at 41°C were
209 significantly down-regulated compared to at 32°C and 37°C (Fig. 2a–d). These results from
210 the mRNA expression analysis are consistent with those of the safranin-O staining and the
211 IHC staining of type II and I collagens (Fig. 3), as well as with the results of the GAG
212 quantification (Fig. 6a), which all indicated that ECM formation was dramatically inhibited at
213 41°C. To elucidate this phenomenon further, we observed the generated pellets at 41°C using
214 SEM to clarify the ultra-microstructure of the collagen, and found that the collagen fiber
215 content in these pellets was very low (Fig. 5). In addition, the results of the DNA
216 quantification suggested that there were significant decreases in the cell number within the
217 pellets at 41°C, by day 14 of culture (Fig. 6b).

218 Peltonen et al.[23] reported that collagen cannot fold into a triple-helix conformation at a
219 temperature of approximately 40°C. Therefore, the reasons for the inhibition of ECM
220 formation at 41°C may be explained by a combination of cell loss, inhibition of ECM-related
221 mRNA expression, and perhaps also by collagen misfolding. Thus, while intermittent heat
222 stimuli may have a positive effect on ECM formation[13,14], prolonged exposure to heat stimuli
223 may have the opposite effect, and we should hence consider heat stimuli as a thermal dose
224 combining both temperature and duration.[24]

225 Interestingly, in this study, the wet weight showed time-dependent increases at 32°C and
226 37°C (Fig. 1b), indicating that a cooler environment (32°C) can resemble ECM produced at
227 37°C, although the wet weight of samples in the 37°C was significantly higher than samples
228 in the 32°C group on days 14 and 21. The wet weight of AC is known to be mainly composed
229 of water (60–85%), type II collagen (15–22%), and proteoglycan (4–7%).[25] As proteoglycan
230 traps water, the most important factor affecting the wet weight is thought to be the
231 proteoglycan content. Herein, we observed a strong correlation between the proteoglycan

232 content and the wet weight (Supplementary Fig. 2); and therefore, the wet weight was
233 thought to be heavier at 37°C due to the higher proteoglycan content (Figs. 3 and 6).
234 Compared to 32°C, from the aspect of the differentiation state, the culture temperature of
235 37°C appeared to enhance redifferentiation of the pellet, which comprised dedifferentiated
236 chondrocytes. Dedifferentiated chondrocytes exhibit increasing fibro-cartilage marker type I
237 collagen and decreasing hyaline-cartilage marker type II collagen.[4,5] In this study, the
238 *COL2A1* mRNA expression (Fig. 2a) and type II collagen protein synthesis (Fig. 4) were
239 higher at 37°C compared to at 32°C. As for the proteoglycan synthesis (Figs. 3 and 6c), the
240 culture temperature of 37°C was also associated with a higher synthesis rate compared to
241 32°C in the late phase (day 21). Therefore, in our experimental condition, the culture
242 temperature of 37°C was able to induce redifferentiation at a higher rate than 32°C, likely by
243 promoting type II collagen synthesis in the early phase and proteoglycan synthesis in the late
244 phase. Taken together, these results suggest that the cells implanted in a patient through ACI
245 are likely to be affected by the intra-articular temperature, and that their growth would be
246 promoted by regulating the temperature at approximately 37°C.
247 The findings from the picrosirius red staining observed under a polarizing microscope
248 showed that integrated collagen fibers were observed in the deep region from day 14 at 32°C
249 (Fig. 3 [white arrow]), whereas it was mainly observed in the superficial region at 37°C.
250 These collagen architectural differences according to the culture temperature are consistent
251 with the findings of previous studies that used immature porcine chondrocytes.[16] Furthermore,
252 upon SEM observation, the collagen fibers in the transition and deep regions also seemed to
253 be denser at 32°C than at 37°C (Fig. 5). In addition, we noted that the compressive response
254 of the pellets was altered by the culture temperature (Supplementary Fig. 3). Although the
255 detailed mechanism of this phenomenon remains unclear, these findings suggest that thermal

environment may affect the function of an articular cartilage. To verify this possibility, further investigations are needed.

Our study has a few limitations. First, the detailed signaling cascade responsible for inducing the differences in chondrocyte metabolism according to the different culture temperatures remains unclear. Second, we investigated only three typical culture temperature conditions. The intra-knee joint temperature in active osteoarthritis and rheumatoid arthritis has been reported to be 34–36°C,[11] and the temperature is altered by the patients' physical activity level.[26] Thus, further investigations are needed to apply multilevel temperature conditions in order to gain an understanding of the precise effects of thermal environment on chondrocytes. Third, we only analyzed cells obtained from two individuals. Therefore, in order to generalize our findings, larger studies are warranted in the future.

In conclusion, the culture temperature of 37°C, which mimics the inner body temperature, was found to promote redifferentiation and ECM formation better than 32°C, which mimics the *in vivo* intra-articular temperature, whereas that of 41°C drastically inhibited ECM formation. Therefore, modulation of thermal environment might represent an advantageous means to regulate the phenotype and biosynthetic activity of chondrocytes. In addition, *in vitro* experiments should consider the culture temperature, since this markedly influences the chondrocyte metabolism and phenotype.

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338

339 ## Figure legends

340 **Figure 1:** Macroscopic observations and wet weight.

341 (a) Representative images of the generated pellets. Scale bar = 1 mm. (b) Wet weight
342 changes of the pellets. The wet weight was heavier at 37°C than at 32°C on days 14 and 21.
343 Values are presented as means ± 95% confidence intervals (n = 9 pellets/group; *P < 0.05,
344 32°C vs. 37°C; **P < 0.01, 32°C vs. 37°C; ‡P < 0.01, 32°C vs. 41°C; §§P < 0.01, 37°C vs.
345 41°C).

346

347 **Figure 2:** Gene expression analysis.

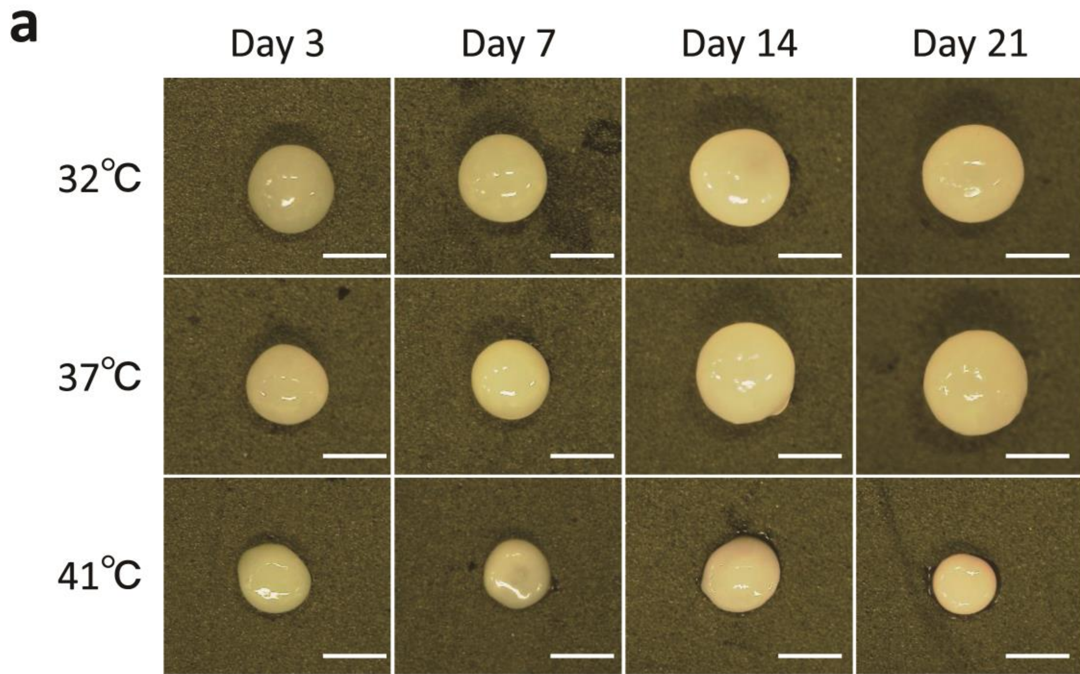
348 Relative mRNA expressions of (a) collagen type II A1 (*COL2A1*), (b) collagen type I A1
349 (*COL1A1*), (c) aggrecan (*ACAN*), and (d) cartilage oligomeric matrix protein (*COMP*) are
350 shown. These genes were up-regulated from days 3 to 7 at 32°C and 37°C, but not at 41°C.
351 *COL2A1* and *COMP* at 37°C were significantly higher than at 32°C on days 3 and 7. There
352 were no significant differences in the *COL1A1* and *ACAN* gene expressions between 32°C
353 and 37°C. The gene expressions at 41°C were all significantly down-regulated. Values are
354 presented as means ± 95% confidence intervals (n = 3 pellets/group; *P < 0.05, 32°C vs.
355 37°C; **P < 0.01, 32°C vs. 37°C; †P < 0.05, 32°C vs. 41°C; ‡P < 0.01, 32°C vs. 41°C; §P <
356 0.05, 37°C vs. 41°C; §§P < 0.01, 37°C vs. 41°C).

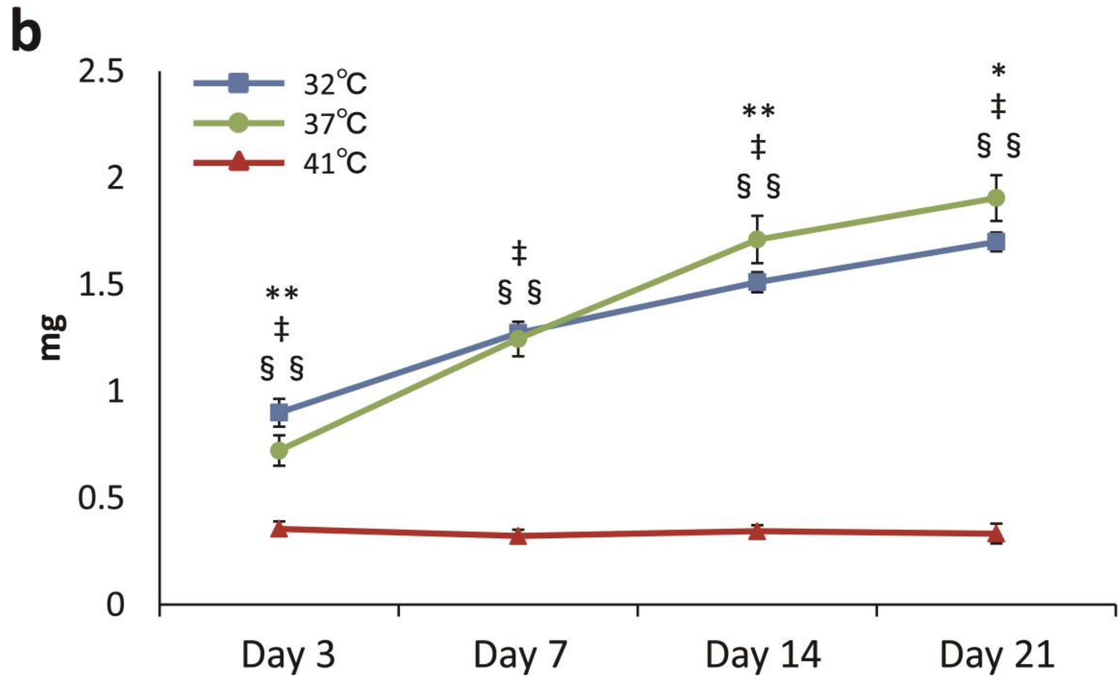
357

358 **Figure 3:** Histological and immunohistochemical analyses.

359 Representative images of the histological and immunohistochemical findings are shown.
360 The staining intensities of safranin-O and type II and I collagen increased over time at 32°C
361 and 37°C, but not at 41°C. Picrosirius red staining demonstrated integrated collagen fibers in

362 the deep region of the pellet at 32°C on days 14 and 21 (white arrow). Scale bar = 500 μm;
363 magnification, ×100.
364
365 **Figure 4:** Semi-quantitative evaluation of type II collagen immunohistochemical staining.
366 (a) The mean type II collagen intensity per pixel and (b) the mean percentage of the type
367 II collagen positive area were calculated on day 21. Both values were higher at 37°C than at
368 32°C. Values are presented as means ± 95% confidence intervals (n = 8 pellets/group; $^{**}P <$
369 0.01).
370
371 **Figure 5:** Scanning electron microscope observations.
372 Representative images of the surface and cut surface of the generated pellets on day 21
373 are shown. The surfaces at 32°C and 37°C, but not at 41°C, appeared to be even and smooth.
374 In the cut surface, dense and layered collagen fibers parallel to the surface were observed in
375 the superficial region at 32°C and at 37°C, but not at 41°C. The collagen fibers in the
376 transition and deep regions at 32°C seemed to be denser than those at 37°C. Scale bar = 10
377 μm; magnification, ×2000.
378
379 **Figure 6:** Glycosaminoglycan (GAG) and deoxyribonucleic acid (DNA) content.
380 (a) The GAG content per pellet at 37°C tended to be higher than that at 32°C, and that at
381 41°C was significantly lower than at the other two culture temperatures. (b) The DNA content
382 per pellet at 41°C was significantly lower than at the other temperature environments. (c) The
383 GAG content normalized by the DNA content (GAG/DNA) was significantly higher at 37°C
384 than at 32°C on day 21. Values are presented as means ± 95% confidence intervals (n = 6
385 pellets/group; $^{**}P < 0.01$).





386

387 **Figure 1**

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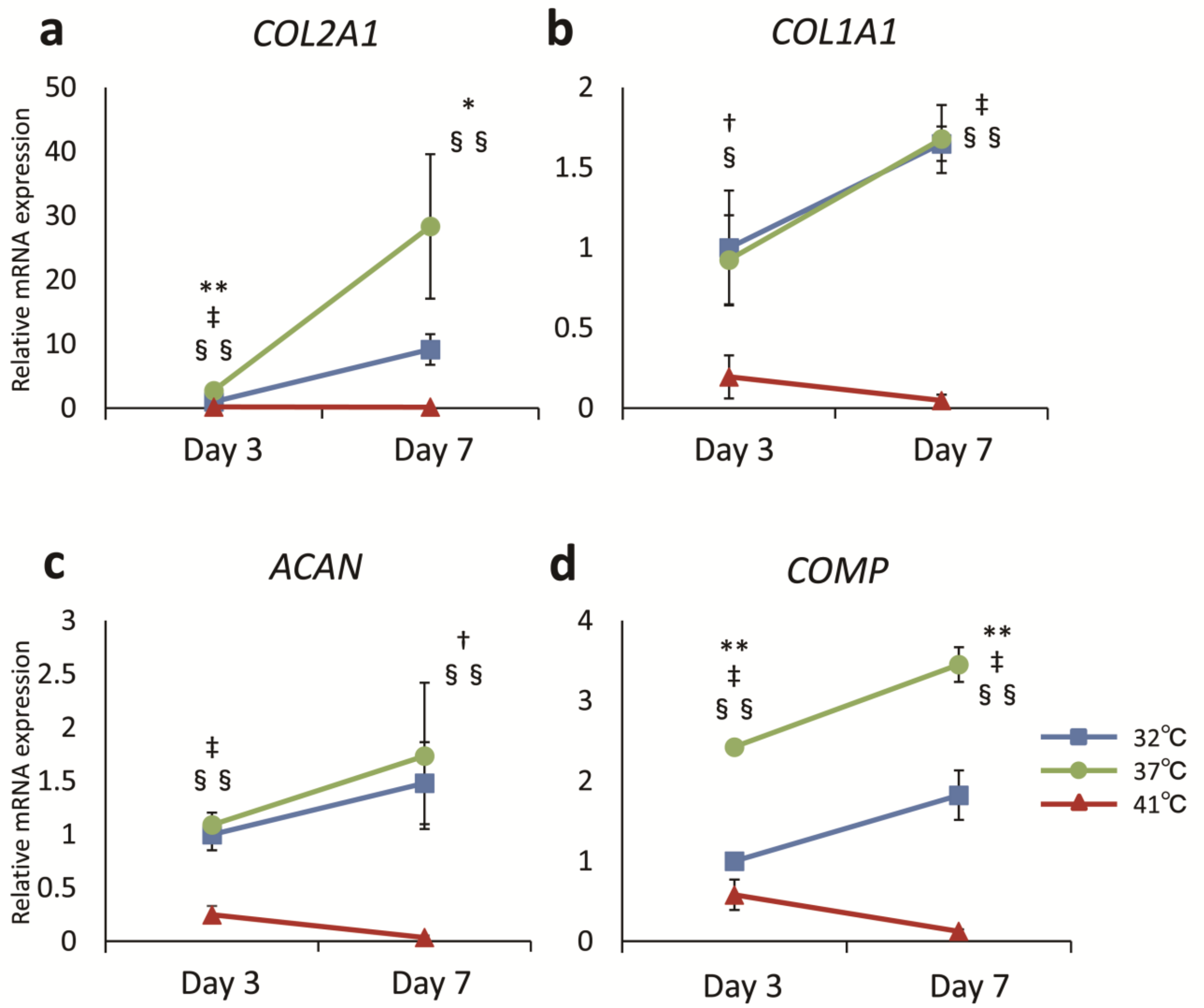

389

390 **Figure 2**

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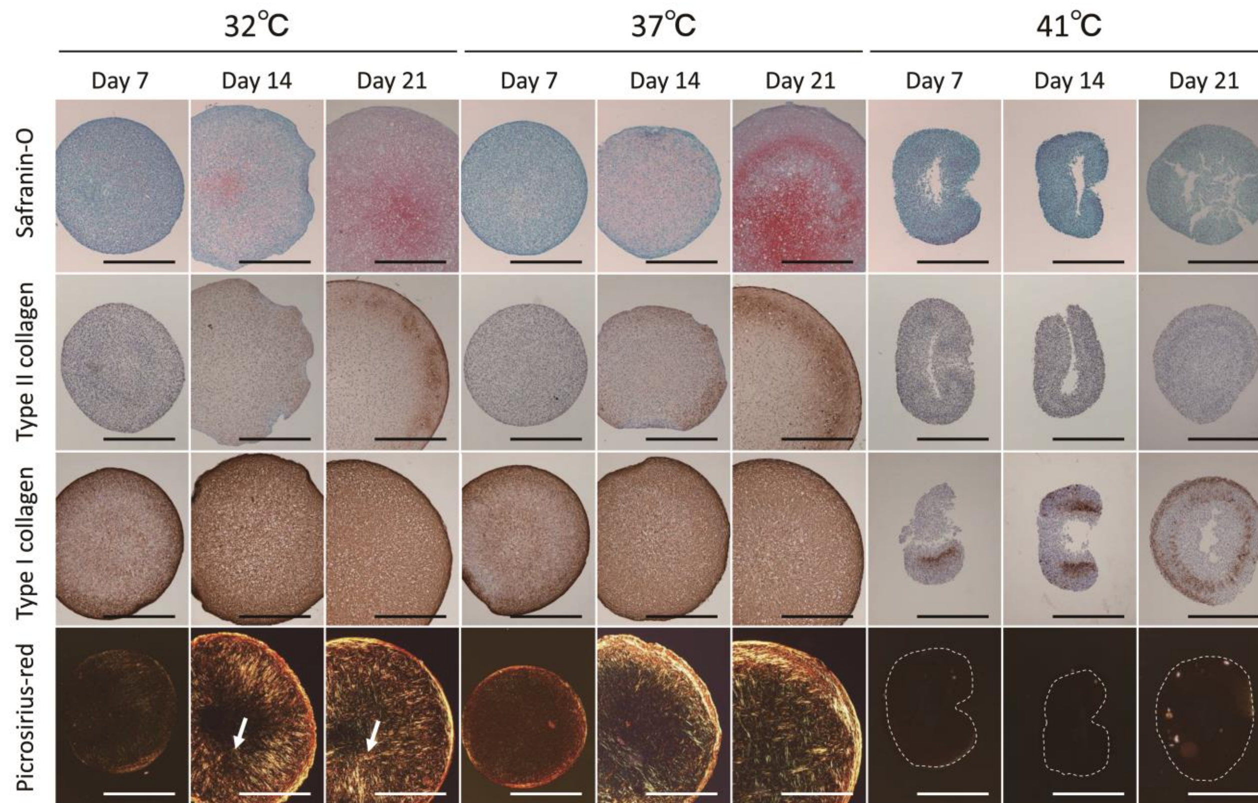

393 **Figure 3**

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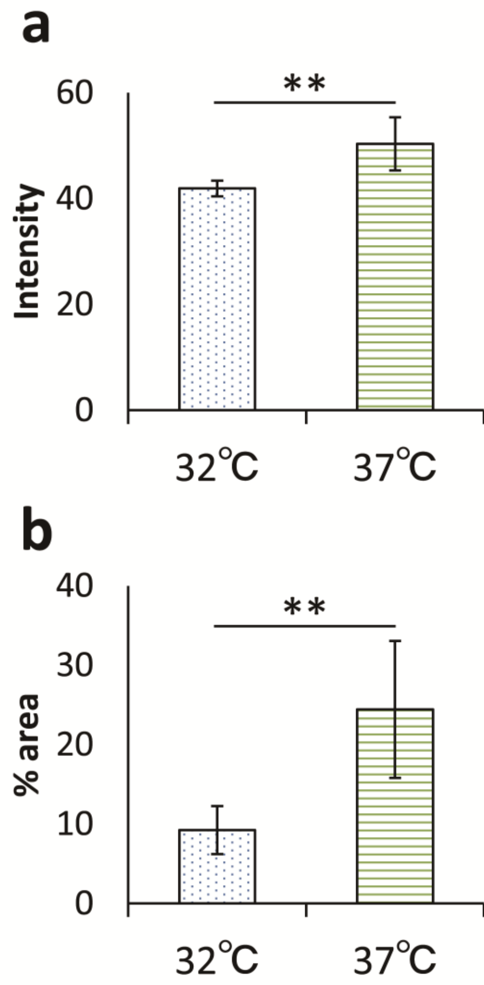

395

396 **Figure 4**

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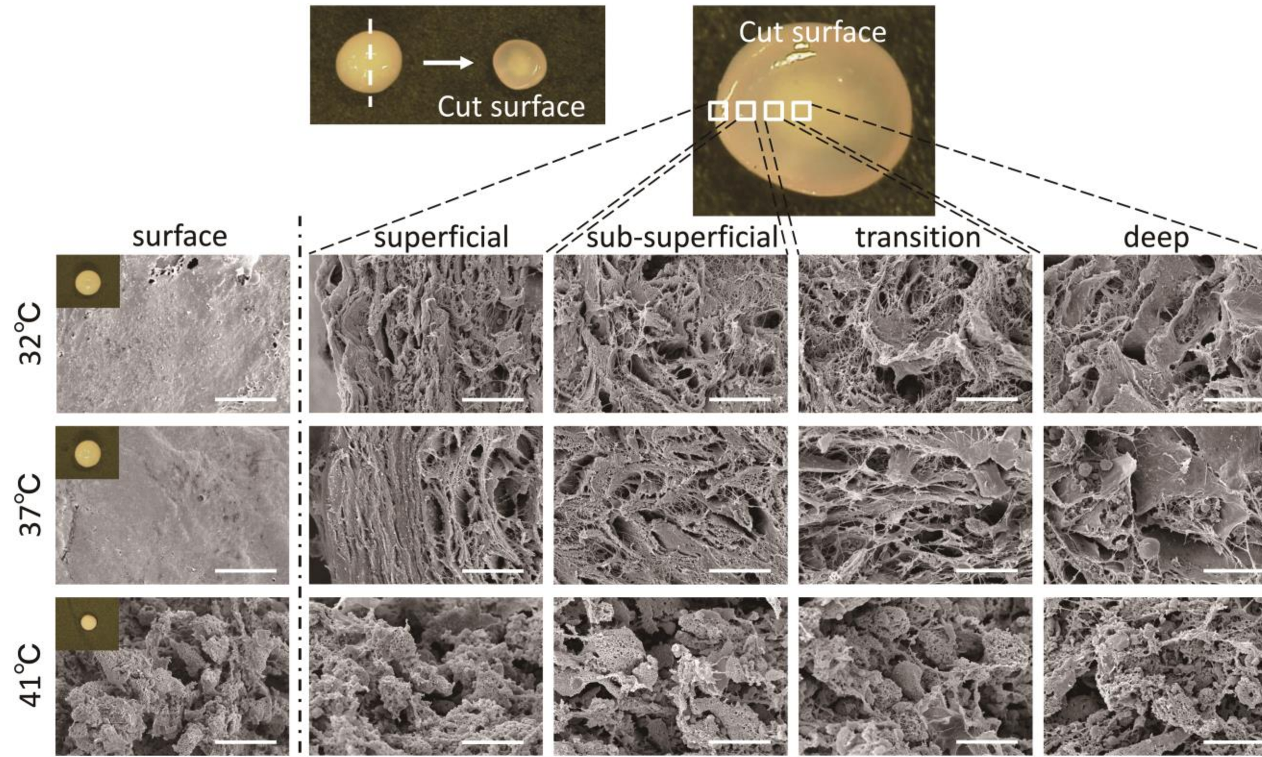

400 **Figure 5**

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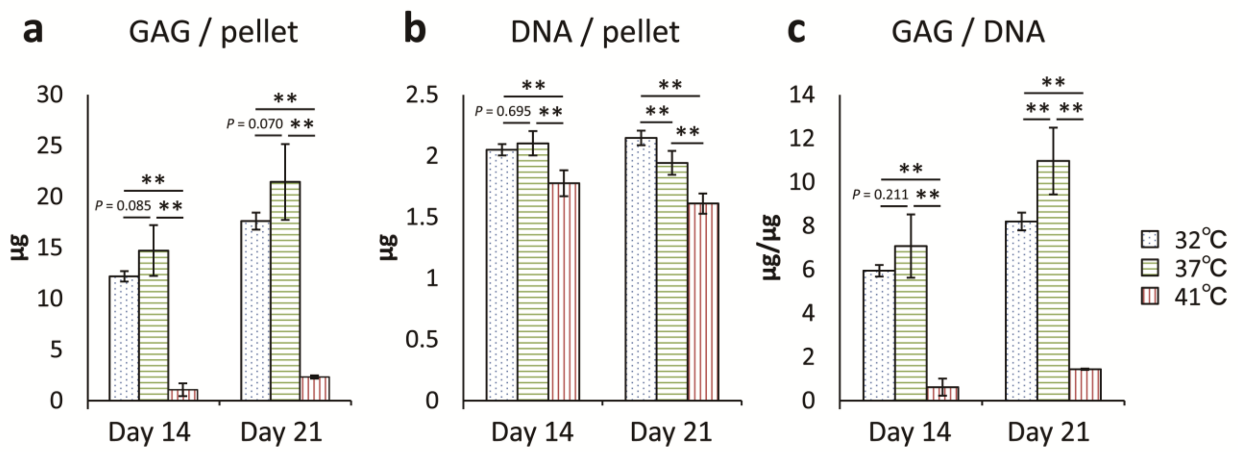
402



403

404 **Figure 6**

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406

Supplementary Table

407

Supplementary Table 1. Primer sequences for qRT-PCR

	Sense (5′–3′)	Antisense (5′–3′)	Length (bp)
COL2A1	GCTATGGAGATGACAACCTGGCTC	CACTTACCGGTGTGTTTCGTGCAG	256
COL1A1	CAGAACGGCCTCAGGTACCA	CAGATCACGTCATCGCACAAC	101
ACAN	GAATTTCCTGGCGTGAGAAC	GGGGATGTTGCGTAAAAGAC	107
COMP	AACAGTGCCCAGGAGGAC	TTGTCTACCACCTTGTCTGC	191
RPL13a	AAGTACCAGGCAGTGACAG	CCTGTTTCCGTAGCCTCATG	100
YWHAZ	TGCTTGCATCCCACAGACTA	AGGCAGACAATGACAGACCA	126

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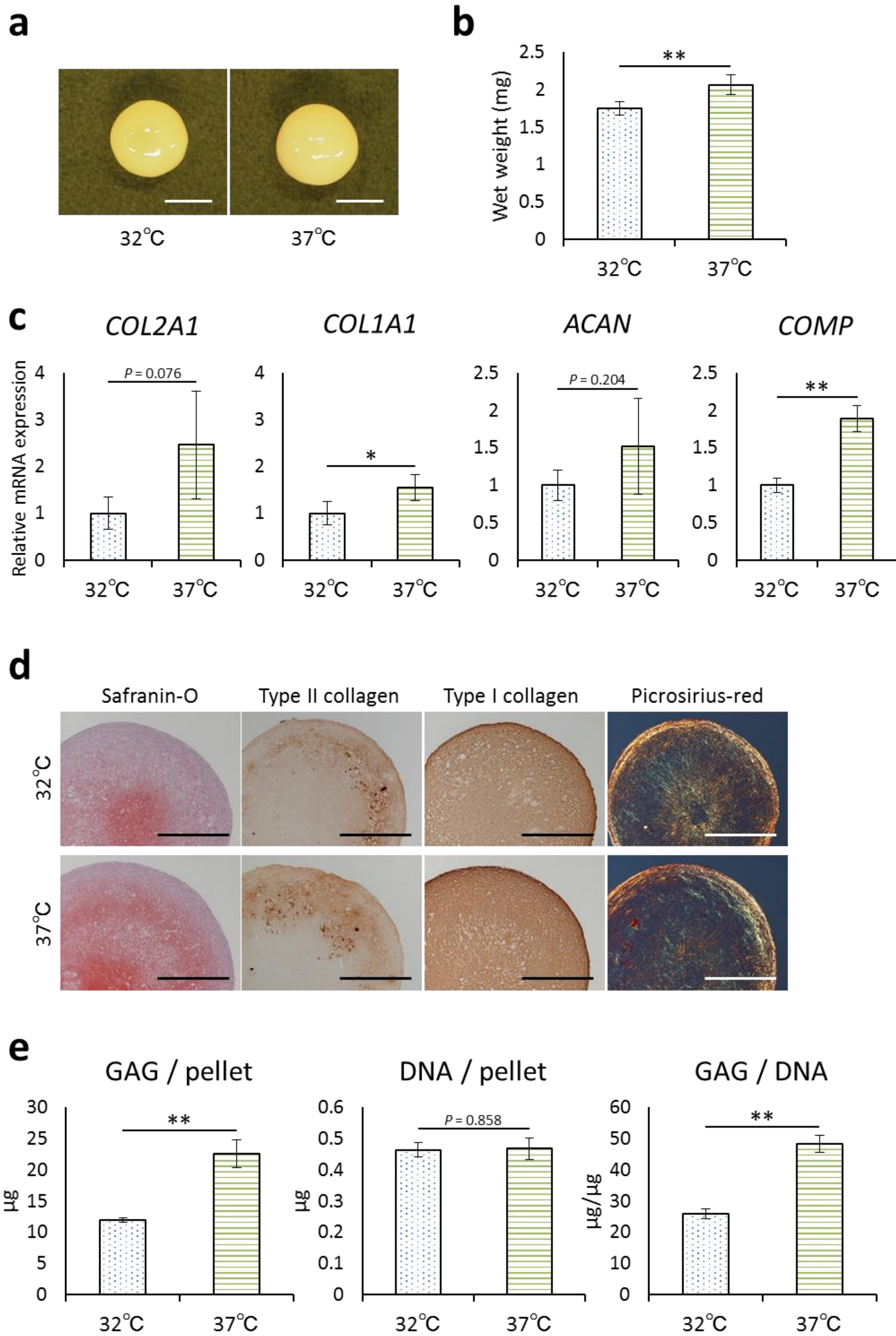
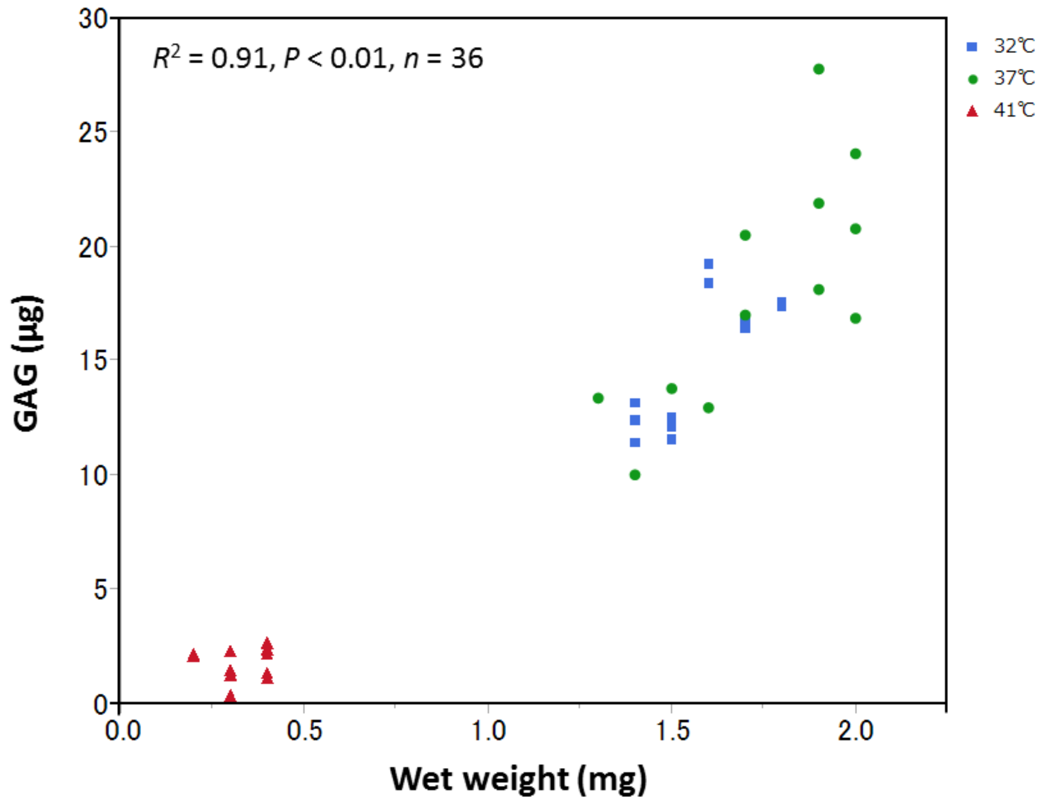


410

411 **Supplementary Figure 1:** Results from donor B.

412 The pellets from donor B cultured at 32°C and 37°C were harvested at 3 and 21 days to
413 confirm the reproducibility of the findings from donor A. (a) Representative images of the
414 generated pellets at 21 days. Scale bar = 1 mm. (b) Wet weight of the pellets at 21 days. The
415 wet weight was heavier at 37°C than at 32°C. Values are presented as means ± 95%
416 confidence intervals (n = 6 pellets/group; $^{**}P < 0.01$). (c) Relative mRNA expressions of
417 *COL2A1*, *COL1A1*, *ACAN*, and *COMP* at 3 days. These genes showed similar trends to those
418 observed for donor A (Fig. 2), except for *COL1A1*. *COL1A1* was slightly, but significantly
419 higher at 37°C than at 32°C. Values are presented as means ± 95% confidence intervals (n = 3
420 pellets/group; $^{*}P < 0.05$, $^{**}P < 0.01$). (d) Representative images of the histological and
421 immunohistochemical findings at 21 days. Similar results as for donor A (Fig. 3) were
422 obtained. Scale bar = 500 μm; magnification, ×100. (e) GAG and DNA content per pellet and
423 the GAG/DNA ratio at 21 days. Similar results as for donor A (Fig. 6) were obtained. Values
424 are presented as means ± 95% confidence intervals (n = 6 pellets/group; $^{**}P < 0.01$).
425 Abbreviations: mRNA, messenger ribonucleic acid; *COL2A1,* collagen type II A1; *COL1A1,*
426 collagen type I A1; *ACAN*, aggrecan; *COMP*, cartilage oligomeric matrix protein; GAG,
427 glycosaminoglycan; DNA, deoxyribonucleic acid.
428



429

430

431 **Supplementary Figure 2:** The correlation between the glycosaminoglycan (GAG) content

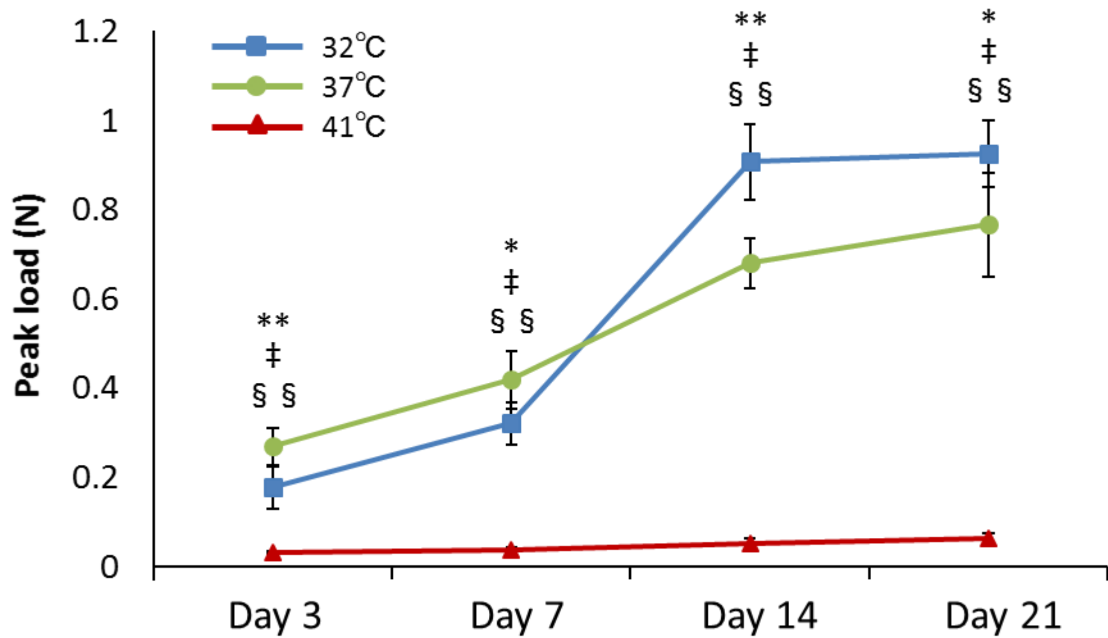
432 and the wet weight.

433 The correlation between the GAG content and the wet weight examined using the

434 Pearson's correlation coefficient has a strong correlation (R^2 = 0.91, P < 0.01, n = 36; blue

435 square, 32°C; green circle, 37°C; red triangle, 41°C).

436



437

438 **Supplementary Figure 3:** Biomechanical analysis.

439 To analyze biomechanical property of the generated pellets cultured at 32°C, 37°C, and
440 41°C, an unconfined compression test was performed using a mechanical testing instrument
441 with the use of a 3-mm diameter indenter (Autograph AG-X; Shimadzu, Kyoto, Japan). Each
442 pellet on days 3, 7, 14, and 21 was compressed uniaxially in a testing chamber filled with
443 PBS at room temperature. A pre-load of 0.01 N was applied and allowed to equilibrate for 60
444 sec. Then the loading was applied at a strain rate of 0.005 mm/s (up to a 50% strain), and
445 peak load (N) was obtained.

446 The peak load was higher at 37°C until day 7. However, after day 14, it was inverted, and
447 the pellets generated at 32°C showed a significantly higher peak load. The peak load at 41°C
448 was significantly lower than others at all days. Values are represented as means ± 95%
449 confidence intervals (n = 6 pellets/group; *P < 0.05, 32°C vs. 37°C; **P < 0.01, 32°C vs.
450 37°C; ‡P < 0.01, 32°C vs. 41°C; §§P < 0.01, 37°C vs. 41°C).