

Peer review information: *Nature Communications* thanks Denis Evseenko and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Engraftment of allogeneic iPS cell-derived cartilage organoid in a primate model of articular cartilage defect

Kengo Abe^{1,2,3}, Akihiro Yamashita^{1,3}, Miho Morioka¹, Nanao Horike^{1,3}, Yoshiaki Takei^{3,4}, Saeko Koyamatsu¹, Keisuke Okita⁵, Shuichi Matsuda², and Noriyuki Tsumaki^{1,3,6,*}

¹Department of Tissue Biochemistry, Graduate School of Medicine and Frontier Biosciences, Osaka University

²Department of Orthopaedic Surgery, Graduate School of Medicine, Kyoto University

³Department of Clinical Application, Center for iPS Cell Research and Application, Kyoto University

⁴Regenerative Medicine Technology Department, Healthcare R&D Center, Asahi Kasei Corporation

⁵Department of Life Science Frontiers, Center for iPS Cell Research and Application, Kyoto University

⁶Premium Research Institute for Human Metaverse Medicine (WPI-PRIME), Osaka University

*To whom correspondence should be addressed:

Noriyuki Tsumaki, M.D., Ph. D.

Department of Tissue Biochemistry, Graduate School of Medicine and Frontier Biosciences, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Tel: +81-6-6879-3321

E-mail: ntsunami@tsu.med.osaka-u.ac.jp

29 **Abstract**

30 Induced pluripotent stem cells (iPSCs) are a promising resource for allogeneic cartilage
31 transplantation to treat articular cartilage defects that do not heal spontaneously and often
32 progress to debilitating conditions, such as osteoarthritis. However, to the best of our
33 knowledge, allogeneic cartilage transplantation into primate models has never been
34 assessed. Here, we show that allogeneic iPSC-derived cartilage organoids survive and
35 integrate as well as are remodeled as articular cartilage in a primate model of chondral
36 defects in the knee joints. Histological analysis revealed that allogeneic iPSC-derived
37 cartilage organoids in chondral defects elicited no immune reaction and directly
38 contributed to tissue repair for at least four months. iPSC-derived cartilage organoids
39 integrated with the host native articular cartilage and prevented degeneration of the
40 surrounding cartilage. Single-cell RNA-sequence analysis indicated that iPSC-derived
41 cartilage organoids differentiated after transplantation, acquiring expression of PRG4
42 crucial for joint lubrication. Pathway analysis suggested the involvement of SIK3
43 inactivation. Our study outcomes suggest that allogeneic transplantation of iPSC-derived
44 cartilage organoids may be clinically applicable for the treatment of patients with
45 chondral defects of the articular cartilage; however further assessment of functional
46 recovery long term after load bearing injuries is required.

47

Introduction

Articular cartilage covers the ends of bones and provides lubrication, which is vital for smooth joint movement and shock absorption. Articular cartilage is avascular and consists of chondrocytes embedded in the extracellular matrix (ECM), which enables the mechanical functions necessary for joint motion and shock absorption. Cartilage ECM consists of collagen fibrils composed of type II, IX, and XI collagen molecules and proteoglycans composed of aggrecan, link protein, and glycosaminoglycans.

As articular cartilage has a limited capacity for repair, and so far, no drugs are available for cartilage repair, focal damage or erosion of articular cartilage frequently leads to debilitating conditions, such as osteoarthritis. Although cell-based therapies have been proposed, only a limited number of autologous chondrocytes are generated since expansion culture steers the chondrocyte character toward that of fibroblastic cells¹, as evidenced in autologous chondrocyte implantation (ACI), wherein more than 90% of the repaired tissue is fibrocartilaginous². Allogeneic cartilage has been transplanted clinically without matching human leukocyte antigen (HLA) types and without the use of immunosuppressive drugs³⁻⁵. However, whether the transplanted allogeneic cartilage causes an immune reaction remains controversial. Some reports suggest low immunogenicity of chondrocytes^{6,7}, whereas others show that chondrocytes are antigenic and elicit varying degrees of immune reactions^{8,9}. To the best of our knowledge, the regenerative mechanisms following allogeneic cartilage transplantation have not yet been reported. Whether transplanted cartilage achieves engraftment (survives and constitutes repaired tissue directly) or only transiently remains and secretes growth factors to stimulate recipient progenitor cells has not been analyzed.

Induced pluripotent stem (iPS) cells are a promising source for the regenerative treatment of articular cartilage damage^{10,11}. Cartilage consisting of chondrocytes and ECM has been successfully created from iPS cells by differentiating them into chondrocytes, which are subsequently transferred into a three-dimensional culture to make iPS cell-derived chondrocytes produce and accumulate ECM around themselves to form cartilaginous tissue particles^{12,13}. Owing to the self-renewal activity of iPS cells, allogeneic iPS cell-derived cartilage organoids can theoretically be produced inexhaustibly and transplanted into an unlimited number of patients, solving the issues

79 associated with allogenic cartilage, such as scarcity of donors, risk of disease transmission,
80 and variations in cartilage qualities between donors.

81 In this study, we analyzed the allogenic transplantation of major
82 histocompatibility complex (MHC)-mismatched iPS cell-derived cartilage organoids in a
83 primate animal model without the use of immunosuppressive drugs. We differentiated
84 *cynomolgus monkey* iPS cells (cyiPSCs) into chondrocytes to create cyiPSC-derived
85 cartilage organoids (cyiPS-Cart). We then transplanted cyiPS-Cart into chondral defects
86 on the knee joint surface of *cynomolgus monkeys* in an allogeneic manner. Single-cell
87 RNA-sequencing (scRNA-seq) and molecular analysis of the cyiPS-Cart graft revealed
88 molecular pathways involved in cell differentiation that remodeled the cyiPS-Cart toward
89 articular cartilage after transplantation.

90

91 **Results**

92 **Preparation of cyiPS cell-derived cartilage organoid (cyiPS-Cart)**

93 1146A1 cyiPSCs expressing enhanced green fluorescent protein (EGFP) under a
94 constitutive promoter were used. Cartilage was created from cyiPSCs¹⁴ and human
95 iPSCs^{12,13} using modified protocols. Briefly, chondrocytes were induced from cyiPSCs
96 in a chondrogenic medium for two weeks and transferred to a three-dimensional culture,
97 where they produced and accumulated ECM to form cartilaginous particles
98 (Supplementary Fig. 1a). The cyiPSC-derived cartilage organoid (cyiPS-Cart) particles
99 were 1–3 mm in diameter (Supplementary Fig. 1b). Histological analysis showed that the
100 particles consisted of cells, and the ECM was stained positively with safranin O.
101 Immunohistochemical analysis revealed that the ECM contained type II collagen
102 (Supplementary Fig. 1c). Type I collagen was not detectable except at the periphery of a
103 particle.

104

105 **Allogeneic transplantation of cyiPS-Cart in primate chondral defect model**

106 Cartilage defects can be classified into two categories based on their depth:
107 chondral defects extending down to but not through the subchondral bone, and
108 osteochondral defects extending down through the subchondral bone (Fig. 1a). Chondral
109 defects are the most common in patients with articular cartilage damage or erosion,
110 including during the early stages of osteoarthritis.

111 We created chondral defects in the femoral trochlear ridge of the right knee joints
112 of 12 *cynomolgus monkeys* and transplanted cyiPS-Cart (transplantation group) in six
113 monkeys or nothing (empty group) in remaining monkeys (Fig. 1b). MHC typing revealed
114 mismatch between cyiPSCs and recipient monkeys (Supplementary Table 1). Computed
115 tomography (CT) imaging analysis of the knee joints indicated that bone structures were
116 normal immediately after surgery and at 4- and 12-week after surgery (Supplementary
117 Fig. 2), suggesting that the defects were chondral and did not extend through the
118 subchondral bone throughout the experiment.

119 Three monkeys from each group were sacrificed 4 and 17 weeks after surgery (n =
120 3). The gross appearance of the joint surface indicated that chondral defects in the empty
121 group were filled with brown tissue at 4 and 17 weeks (Fig. 1c). On the other hand,

122 chondral defects in the transplantation group were filled with transparent tissue at 4 weeks
123 (Fig. 1c) which later turned white as articular cartilage, making it difficult to distinguish
124 the area where cyiPS-Cart were transplanted from the surrounding articular cartilage area
125 at 17 weeks after transplantation (Fig. 1c). In each monkey, one transplant site was
126 harvested and subjected to histological analysis and other two sites were combined and
127 used for scRNA-seq analysis.

128

129 **Allogeneic transplantation of cyiPS-Cart did not elicit an immune reaction in** 130 **primate chondral defects**

131 We recently reported that allogeneic cyiPS-Cart elicited an immune reaction when
132 transplanted into osteochondral defects¹⁴. However, no scientific evidence exists on
133 whether allogeneic cartilage elicits an immune reaction when implanted in chondral
134 defects. To answer this question, we analyzed histological sections of the transplanted
135 sites. For control, we created osteochondral defects in additional three monkeys,
136 transplanted cyiPS-Cart, and sacrificed them 4 weeks later.

137 Four weeks after allogeneic transplantation into osteochondral defects, histological
138 analysis revealed that many cells, including CD3+ T lymphocytes, accumulated around
139 the cyiPS-Cart in the bone marrow (Fig. 2 a, b), which is consistent with a previous
140 report¹⁴. In contrast, there was no cell accumulation around chondral defects transplanted
141 with allogeneic cyiPS-Cart (Fig. 2 a, b).

142

143 **cyiPS-Cart survived and directly contributed to hyaline cartilage-rich repaired** 144 **tissue in chondral defects.**

145 The quality of repaired tissues in the chondral defects was assessed by staining
146 histological sections with safranin O, which stains the cartilaginous proteoglycans.
147 Chondral defects in the empty group were partially filled at 4 weeks and substantially
148 filled at 17 weeks after surgery, with tissues that were not cartilaginous but fibrous, as
149 indicated by negative safranin O staining (Fig. 3a; Supplementary Fig. 3 and 4a). In
150 contrast, chondral defects in the transplantation group were filled with cartilaginous tissue
151 both at 4 and 17 weeks after surgery, as indicated by positive safranin O staining (Fig. 3a;
152 Supplementary Fig. 3 and 4a).

153 Repaired tissues formed in the chondral defects in the empty groups at both 4- and
154 17 -weeks after transplantation showed positive picosirius red staining under polarized
155 microscopy (Fig. 3a), suggesting that the repaired tissues were fibrous. On the other hand,
156 marginal staining was observed in the repaired tissues that filled chondral defects and the
157 articular cartilage in the transplantation groups at both 4- and 17- weeks after
158 transplantation (Fig. 3a), suggesting that they were hyaline cartilage.

159 Scoring cartilage repair (Supplementary Table 2) revealed better cartilage
160 regeneration in the transplantation group than in the empty group (Fig. 3b). The score
161 improved at 17 weeks from 4 weeks after transplantation (Fig. 3b).

162 The articular cartilage adjacent to the chondral defect in the empty group lost
163 safranin O staining at 17 weeks after surgery (Fig. 3a, *arrows*; Supplementary Fig. 4b).
164 The remaining cartilage that locates between the bottom of the defect and bone also lost
165 safranin O staining at 4 and 17 weeks after surgery in the empty group (Fig. 3c, area
166 below the *dotted lines*). These results indicate progressive degeneration of the articular
167 cartilage around the defects. In contrast, the articular cartilage surrounding the chondral
168 defect maintained proteoglycan in the transplantation group at 17 weeks after surgery,
169 suggesting preservation of articular cartilage around the defects (Fig. 3a, *arrow heads*;
170 Fig. 3c, area below the *dotted lines*; Supplementary Fig. 4b).

171 Immunostaining with anti-GFP antibody revealed that almost all cells in the
172 repaired tissues that filled the chondral defects in the transplantation group expressed GFP
173 (Fig. 4) at both 4 and 17 weeks after transplantation, indicating that transplanted cyiPS-
174 Cart survived and directly contributed to the entire repaired tissue for at least 4 months.
175 Additional immunostaining with antibodies that recognize type I collagen (COL1), a
176 marker for fibrous tissue, and type II collagen (COL2), a marker for cartilage, confirmed
177 that the surviving cyiPS-Cart filling chondral defects contained hyaline cartilage. In
178 contrast, the tissues filling empty chondral defects were fibrous (Fig. 4).

179 The integration of repaired tissue and surrounding native cartilage is difficult,
180 especially in the case of chondral defects, because chondral defects do not bleed¹. In our
181 study, cyiPS-Cart did not achieve integration at 4 weeks, but integration was observed 17
182 weeks after transplantation. Although immature tissue was still bridging the cyiPS-Cart

183 and surrounding native cartilage, it was mainly composed of type II collagen (Fig. 4,
184 *arrows*).

185 No accumulation of immune cells, including CD3⁺ T lymphocytes, was observed
186 in the transplantation group 17 weeks after allogeneic transplantation into osteochondral
187 defects (Fig. 3a, 4b), indicating that allogeneic transplantation of cyiPS-Cart into chondral
188 defects did not elicit immune reactions in the primate model for at least 4 months.

189

190 **Post-transplant cyiPS-Cart remains cartilaginous while tissues formed in chondral**
191 **defects in the empty group were fibrous.**

192 To investigate the fate of cyiPS-Carts after transplantation, we performed scRNA-
193 seq analysis. Single cells were prepared from undifferentiated cyiPSCs (cyiPSC), cyiPS-
194 Cart (pre-transplant cyiPS-Cart), intact articular cartilage (cyAC), fibrous tissue formed
195 in chondral defects in the empty group (cyFT), and cyiPS-Cart in chondral defects in the
196 transplantation group (post-transplant cyiPS-Cart) 17 weeks after surgery (Fig. 5a).

197 As for chondrogenic differentiation of cyiPSCs toward pre-transplant cyiPS-Cart,
198 scRNA-seq analysis revealed that cyiPSCs expressed pluripotency markers, whereas cells
199 in pre-transplant cyiPS-Cart did not express pluripotent markers but expressed
200 chondrocyte markers (Fig. 5b). Some of the pre-transplant cyiPS-Cart cells expressed
201 *COL1A1* (Fig. 5b) and likely resided in the periphery of the pre-transplant cyiPS-Cart, as
202 indicated by COL1 immunostaining (Supplementary Fig. 1c). All cyiPSCs and pre-
203 transplanted cyiPS-Cart cells expressed EGFP. The uniform manifold approximation and
204 projection (UMAP) plot indicated that cyiPSCs and pre-transplanted cyiPS-Cart cells
205 were plotted as separate clusters (Fig. 5c). Featureplot analysis revealed that the pre-
206 transplant cyiPS-Cart cell cluster expressed chondrocyte markers but not pluripotent
207 markers (Fig. 5d), indicating chondrogenic differentiation of cyiPSCs into pre-transplant
208 cyiPS-Cart cells.

209 For samples harvested at 17 weeks after surgery, VlnPlot function (Seurat) revealed
210 positive GFP expression in almost all cells in the post-transplant cyiPS-Cart (Fig. 6a),
211 confirming that cyiPS-Cart survived and directly contributed to the repaired tissue.
212 Almost all cells in post-transplant cyiPS-Cart expressed *COL2A1* and not *COL1A1*,
213 whereas the majority of cells in cyFT expressed *COL1A1* but not *COL2A1* (Fig. 6a),

214 confirming that post-transplant cyiPS-Cart remained hyaline cartilaginous while the cyFT
215 was fibrous.

216 Then, we analyzed cell subpopulations in cyAC, cyFT, pre-transplant cyiPS-Cart
217 and post-transplant cyiPS-Cart samples. We reduced cell numbers to 320 in each sample
218 using subset function, integrated samples into single object¹⁵. We also reduced
219 dimensions, clustered the cells with a parameter resolution of 0.2, and projected them
220 onto a UMAP plot (Fig. 6b). Cell clustering analysis revealed that cyAC, pre-transplant
221 cyiPS-Cart, and post-transplant cyiPS-Cart had similar transcriptional profiles, whereas
222 FT contained cell clusters with distinct profiles. cyAC, pre-transplant cyiPS-Cart, and
223 post-transplant cyiPS-Cart were composed of cluster # 0, whereas FT was composed of
224 clusters # 1 and #2 (Fig. 6c, d). Cells in clusters #0 and #2 exhibited high expression of
225 *COL2A1* whereas those in cluster #1 highly expressed *COL1A1* (Fig. 6e). Differentially
226 expressed genes (DEGs) were identified (Fig. 6f). Canonical pathway analysis based on
227 the DEGs indicated that cluster #1 was enriched for the fibrosis pathway and that cluster
228 # 2 was enriched for the osteoarthritis pathway (Fig. 6g). These results suggest that
229 clusters #1 and #2 consisted of pathological cells and contain few cyAC, pre-transplant
230 cyiPS-Cart, and post-transplant cyiPS-Cart cells (Fig. 6d). We further compared these
231 cell clusters with those previously identified in human osteoarthritis samples¹⁶. The
232 expression of marker genes for osteoarthritis¹⁶ in our clusters suggested that cluster #1
233 corresponds to preHTC and FC (high expression of *TGFBI* and *COL1A1*) and that cluster
234 #2 corresponds to EC and proC (high expression of *TF* and *P3H2*) (Supplementary Fig.
235 5 a,b).

236 To analyze the resemblance and difference between cyAC and post-transplant
237 cyiPS-Cart cells, we selected these cells and performed clustering analysis again. Cells
238 were divided into four clusters (Supplementary Fig. 6 a,b). Clusters #0, 1 and 2 consisted
239 of both cyAC and post-transplant cyiPS-Cart cells, whereas post-transplant cyiPS-Cart
240 cells were excluded from cluster #3 (Supplementary Fig. 6 a, c, and d). DEGs and
241 canonical pathway analysis indicated that cluster #3 was enriched for integrin signaling
242 (Supplementary Fig. 6 e,f). Trajectory inference and RNA velocity analysis suggested
243 that cluster #3 was located at the start of the trajectory to #0 (Supplementary Fig. 6g),
244 whereas cluster #2 was located at the end. These results suggest that post-transplant

245 cyiPS-Cart are similar to cyAC except for cells from cluster #3 that are related to integrin
246 signaling and locate at the start of the trajectory.

247

248

249 **cyiPS-Cart became similar to articular cartilage after transplantation, with cells**
250 **differentiated to express *PRG4*, achieving engraftment.**

251 Next, we examined how the nature of cyiPS-Cart was altered after transplantation
252 into a chondral defect. Among the differentially expressed genes between pre-and post-
253 transplant cyiPS-Cart, the expression of gene encoding proteoglycan 4 (*PRG4*) was
254 significantly increased (adjusted p-value = 1.55×10^{-28}) (Fig. 7a). *PRG4* is expressed in
255 the superficial zone of articular cartilage and crucial for the lubrication of the joint
256 surface¹⁷⁻¹⁹. The VlnPlot function (Seurat) revealed that few cells expressed *PRG4* in pre-
257 transplant cyiPS-Cart, while a substantial number of post-transplant cyiPS-Cart cells
258 showed high-level *PRG4* expression compared to cyAC cells (Fig. 7 b). FeaturePlot
259 function (Seurat) confirmed that few cells in the pre-transplant cyiPS-Cart expressed
260 *PRG4* whereas many cells in the post-transplant cyiPS-Cart expressed *PRG4* (Fig. 7c).
261 Immunohistochemical analysis revealed that *PRG4* expression was hardly detected in
262 pre-transplant cyiPS-Cart, consistent with the low expression of *PRG4* mRNA observed
263 in scRNA-seq analysis (Fig. 7d). In contrast, post-transplant cyiPS-Cart expressed *PRG4*,
264 and its expression was localized to the superficial zone of post-transplant cyiPS-Cart. This
265 expression pattern was consistent with that in cyAC (Fig. 7d).

266 To gain further insights into the mechanism by which cyiPS-Cart acquired *PRG4*
267 expression, we analyzed scRNA-seq data. We combined pre-and post-transplant cyiPS-
268 Cart cells and separated them into two groups: cells whose *PRG4* expression was more
269 than or equal to 2.4, and those whose *PRG4* expression was less than 2.4. We then
270 identified differentially expressed genes (DEGs) between the two groups using the
271 FindMarkers function (Seurat) (Supplementary Data 1) and subjected the DEGs to
272 Ingenuity Pathway Analysis (IPA, Qiagen). IPA detected possible upstream regulators,
273 including activated TGF- β 1, activated TGF- β 3, activated SMAD3, activated TGF- β ,
274 activated TGF- β 2, and inhibited SMAD7 (Supplementary Data 2). The addition of TGF-
275 β upregulates *Prg4* expression in chondrocytes^{20,21}. The addition of TGF- β 1 to the culture

276 of cyiPS-Cart cells increased *PRG4* mRNA expression (Fig. 7e), whereas TGF- β inhibitor
277 downregulated *PRG4* mRNA expression (Fig. 7e). These results suggest that the TGF- β
278 signaling pathway is involved in *PRG4* activation in cyiPS-Cart after transplantation.

279 IPA also detected forskolin as an upstream activator of genes whose expression
280 was upregulated in PRG4-positive cells in cyiPS-Cart (Supplementary Data 2). Forskolin
281 upregulates *PRG4* expression in chondrocytes by increasing the concentration of cAMP,
282 which activates PKA and CREB²². In contrast, forskolin inactivates salt-inducible kinase
283 2 (SIK2) via the PKA-dependent phosphorylation of SIK2 in hepatocytes²³. Thus, we
284 hypothesized that SIK is involved in regulating *PRG4* expression and performed further
285 experiments. Among the SIK family members, SIK3 mainly functions in chondrocytes²⁴⁻
286 ²⁶. Forskolin increased the amount of Sik3 phosphorylated at threonine 411 (pSIK3
287 (pT411), an inactive form of Sik3 (Fig. 8a)) and increased *Prg4* expression (Fig. 8b) in
288 murine chondrocytes, indicating an association between Sik3 activity and *Prg4*
289 expression. *Prg4* mRNA expression increased in primary chondrocytes obtained from
290 Sik3 knockout mice but decreased in primary chondrocytes obtained from transgenic
291 mice overexpressing *Sik3* in chondrocytes (Figure 8c). Immunohistochemical analysis
292 showed that the population of chondrocytes expressing Prg4 increased in Sik3 conditional
293 knockout mice (Figure 8d; Supplementary Fig. 7a). These results suggested that Sik3
294 inhibits *Prg4* expression. *In vivo*, the joint surface is subjected to fluid flow shear stress
295 (FFSS), inducing the expression of *Prg4* gene²². To mimic this *in vivo* situation, FFSS
296 was applied to mouse primary chondrocytes (Supplementary Fig. 7b). Application of
297 FFSS to wild-type mouse primary chondrocytes increased the expression of *Prg4* after 12
298 h (Fig. 8e; Supplementary Fig. 5c, open circles). *Sik3* deletion further increased FFSS-
299 induced *Prg4* expression (Fig. 8e; Supplementary Fig. 7c, closed circles), suggesting that
300 Sik3 helps regulate *Prg4* expression in mouse chondrocytes. The induction of *PRG4*
301 indicates that the post-transplant cyiPS-Cart acquired lubrication function as articular
302 cartilage.

303 We analyzed the relationship between TGF- β and Sik3 inactivation. The addition
304 of TGF- β did not affect phosphorylation of Sik3 at T411 (Supplementary Fig. 8), whereas
305 the addition of forskolin increased phosphorylation of Sik3 at T411 but did not affect the

306 phosphorylation of Smad3 (Supplementary Fig. 8). These results suggest that TGF- β and
307 *Sik3* regulate *Prg4* expression independently.

308 The restricted expression of PRG4 in the superficial zone of the post-transplant
309 cyiPS-Cart suggests that cyiPS-Cart after transplantation survived and directly
310 contributed to repair tissue in chondral defects, and also functioned as articular cartilage.
311 This is further supported by the result that the transplantation of cyiPS-Cart prevented the
312 degeneration of the native articular cartilage surrounding chondral defects. These results
313 collectively indicate the successful engraftment of allogeneic cyiPS-Cart in a chondral
314 defect in the knee joints of the primate model.

315

316 **Discussion**

317 To the best of our knowledge, this is the first study to provide scientific evidence
318 in a primate model that allogeneic cartilage achieves engraftment in chondral defects,
319 without inducing immune reactions. In contrast, allogeneic cyiPS-Cart elicits an immune
320 reaction in osteochondral defects¹⁴. Cartilage is believed to be relatively
321 immunoprivileged^{6,27} because chondrocytes are surrounded by the ECM, which protects
322 the chondrocytes from exposure to cells involved in immunological reactions. The lack
323 of an immune reaction following cyiPS-Cart transplantation in chondral defects, which
324 bleed minimally, suggests that exposure of allogeneic cyiPS-Cart in osteochondral
325 defects to abundant blood flow in the bone marrow might elicit an immune reaction
326 despite the presence of ECM. Although allogeneic cyiPS-Cart in osteochondral defects
327 survives, further studies are needed to determine whether the degree of the immune
328 reaction is tolerable for effective regeneration. While it remains to be determined whether
329 osteochondral defects can be cured by allogeneic cartilage transplantation²⁸, our results
330 indicate that chondral defects can be a definite indication for allogeneic cartilage
331 transplantation.

332 Most cell-based therapies induce cartilage regeneration through a mechanism
333 called trophic effects, wherein implanted cells survive only transiently and secrete growth
334 factors that stimulate the host progenitor cells. Evidence that implanted cells achieve
335 engraftment and directly contribute to tissue repair is scant²⁹⁻³¹. In a recent study human
336 embryonic stem cell (ESC)-derived chondrocytes that do not associate with cartilage
337 ECM were implanted into the articular cartilage defects of mini-pigs³². Of the cells that
338 formed in the repaired tissue in the defects, 4% were human, indicating that most of the
339 cells that form in repair tissue are those of the recipient. Although experimental conditions
340 of the two studies differ (xenograft vs. allograft; critical vs. small size defects; 6 vs. 4
341 months' observation), allogeneic cyiPS-Cart survived for at least four months, and almost
342 all cells in the repair tissue were transplanted cells, as demonstrated by GFP expression
343 in our study. In addition, cyiPS-Cart cells differentiated into articular chondrocytes after
344 transplantation and functioned as articular cartilage. Our results suggest that SIK3 could
345 be involved in post-transplant differentiation. Although we do not know whether this
346 differentiation mechanism is specific to cyiPS-Cart, we speculate that two specific

347 features of iPS-Cart could favor its cells to differentiate and survive: First, the iPS-Cart
348 is composed of chondrocytes and cartilage ECM, which contribute to the survival and
349 differentiation of chondrocytes by providing an appropriate environment for
350 chondrocytes. Second, iPS-Cart has characteristics of embryonic cartilage³³ that would
351 contribute in its survival and differentiation. The survival and differentiation capacity of
352 cyiPS-Cart enables a new strategy whereby damaged cartilage can be replaced with
353 transplanted cartilage. Regarding clinical relevance, it has not been known whether
354 engraftment of cartilage transplants gives better clinical results, such as improved joint
355 function and pain relief, than repair tissue formed by trophic effects or vice versa. It is
356 plausible that engraftment of cartilage transplants is better indicated for severe cartilage
357 lesion where the provision of host progenitor cells is limited. Further study is required to
358 clarify the indications.

359 The treatment of chondral defects is challenging, particularly in two aspects. First,
360 the chondral defects do not bleed and, therefore, do not initiate the wound healing process.
361 Therefore, chondral defects are occasionally treated by microfracture surgery, wherein
362 the subchondral bone is invasively pierced to introduce mesenchymal cells into the defect.
363 Cell-based therapies combined with microfracture probably initiate a regenerative
364 mechanism by which implanted cells secrete factors that stimulate mesenchymal cells in
365 the bone marrow, which in turn improves tissue repair. However, the repair tissue remains
366 fibrous because the chondrogenic capacity of host progenitor cells is limited. The
367 regenerative mechanism of survival and replacement by cyiPS-Cart does not require an
368 invasive procedure for microfracture because it heals the defect independently of host
369 progenitor cells.

370 Second, the integration between the repaired tissue and the surrounding native
371 cartilage is hardly achieved^{1,34,35}. Thus, the integration of cyiPS-Cart with host cartilage
372 is promising. Human iPSC-derived cartilage organoids have the capacity for integration³⁶.
373 FGF signals are involved in this integration. Further studies are needed to fully understand
374 this integration mechanism and to enhance the integration of cartilage grafts.

375 There are several limitations to this study. First, the *cynomolgus monkeys* used in
376 this study are not large animals, which makes it difficult to reproduce changes in
377 biomechanics observed in cartilage lesions in humans. Although chondral defects in the

empty group were filled with fibrous tissue in a four-month interval, the small size of the defects would eventually heal after a longer period. Hence, our results will need to be confirmed in larger animal models with critical-sized defects. In addition, we did not analyze the biomechanical properties of post-transplant cyiPS-Cart. Biomechanical test will be required to determine clinical relevance. Second, an observation period of 17 weeks (4 months), as employed in this study, is not long enough to demonstrate the sustainability of a transplant. It is, however, still possible to conclude that engrafted cells had obtained a degree of articular chondrocyte identity by 4 months. A longer observation period, such as two years, would further signify the sustainability and turnover of transplants. Third, although our results suggest that *Sik3* inhibits *Prg4* expression in mouse chondrocytes, this does not prove that SIK3 is involved in *PRG4* expression in post-transplant cyiPS-Cart in monkeys. In conclusion, cyiPS-Cart transplanted into chondral defects survived, integrated with native cartilage, acquired *PRG4* expression in the superficial region, and prevented degeneration of the surrounding cartilage. These results collectively suggest that allogeneic cyiPS-Cart engraftment will contribute to the development of translational medical techniques based on allogeneic pluripotent stem cells to treat chondral defects in articular cartilage.

396 **Methods**

397 **Ethics statement**

398 All methods were performed following relevant guidelines and regulations.
399 Experiments using recombinant DNA were approved by the Recombinant DNA
400 Experiments Safety Committees of Kyoto University (No. 180041) and Osaka University
401 (No. 04794). All animal experiments were approved by the Institutional Animal
402 Committees of the Kyoto University (No. 18-101-14 and No. 16-74-17) and Osaka
403 University (No. 03-044-014).

405 **Isolation of *cynomolgus* monkey iPSCs and creation of cyiPS-Cart by chondrogenic** 406 **differentiation of cyiPSCs**

407 We prepared a cyiPSC line, 1146A1, in which the EGFP gene was integrated into the
408 AAVS1 locus using the pBS-macAAVS1-P-CAG-GFP vector and CRISPR-Cas9 system.
409 The cyiPSC lines had homozygous MHC haplotypes (Mafa-HT1; Mafa is the MHC of a
410 *cynomolgus macaque*)³⁷.

411 The cyiPSCs were maintained on a mitomycin C-inactivated feeder layer of mouse
412 embryonic fibroblasts (MEF) in Dulbecco's Modified Eagle Medium/ Ham's F12
413 (DMEM/F12) medium (Sigma) containing 20% knockout serum replacement (KSR;
414 Thermo Fisher Scientific), 100 μ mol/L 2-mercaptoethanol (Thermo Fisher Scientific), 1
415 $\times 10^{-4}$ M nonessential amino acids (Thermo Fisher Scientific), 1 mM sodium pyruvate
416 (Thermo Fisher Scientific), 2 mM GlutaMAX (Thermo Fisher Scientific), and 50 units
417 penicillin and 50 mg/mL streptomycin (1% PC/SM, Thermo Fisher Scientific). The
418 medium was changed every day. Every 7 days, the cyiPSC colonies were subjected to
419 0.1% collagenase treatment for 3 min (Thermo Fisher Scientific), and cells were collected,
420 centrifuged, and replated onto dishes with new MEF feeder cells³⁸.

421 For chondrogenic differentiation, all cyiPSCs were loosely detached from the MEF
422 feeder cells by exposure to 0.1% collagenase and cultured in Stemfit AK02N medium
423 (Ajinomoto, Tokyo, Japan) on Matrigel-coated dishes for 7 days. CyiPSCs were
424 differentiated into chondrocytes, and cartilage was formed using a previously described
425 method for human iPSCs^{12,13}. Briefly, after chondrogenic differentiation, the cells were
426 transferred into suspension culture to form cartilaginous particles 1-3 mm in diameter.

427 Differentiated chondrogenic cells and particles were cultured in chondrogenic medium
428 (DMEM [Sigma] with 1% ITS-X [Thermo Fisher Scientific], 1% FBS [Thermo Fisher
429 Scientific], 1×10^{-4} M nonessential amino acids, 1 mM sodium pyruvate, 1% PC/SM, 50
430 $\mu\text{g/mL}$ ascorbic acid [Nacalai Tesque], 10 ng/mL BMP2 [Peprotech], 10 ng/mL TGF β 1
431 [Peprotech], and 10 ng/mL GDF5 [BioVision]). We also created cyiPS-Cart from the
432 previously reported cyiPSC line, 1231C1-G³⁹.

433

434 **Transplantation of cyiPSC-derived cartilage organoid into osteochondral or** 435 **chondral defects in *cynomolgus monkey***

436 *Cynomolgus monkeys* (3-4 years old) were purchased from Ina Research (Nagano,
437 Japan). Under general anesthesia, the skin and joint capsules of the right knee were
438 opened in 12 monkeys. Chondral defects (1 mm diameter and 0.5 mm depth) were created
439 at the trochlea of the distal femur under surgical microscopy. We used the electric router
440 (Dremel, micro, please see figure below) and a dental steel bur under a surgical loupe to
441 create consistent chondral defects. We transplanted 1146A1 cyiPSC-derived cyiPS-Cart
442 into the chondral defects by press-fitting in six monkeys (transplantation group). We
443 transplanted nothing into the other six monkeys (empty group). The joint capsule and the
444 skin were closed. After surgery, we intramuscularly injected antibiotics and
445 buprenorphine hydrochloride (0.1 mg/body) for three days into all monkeys.

446 Three monkeys in each group were sacrificed either 4 or 17 weeks after injecting
447 pentobarbital sodium (100 mg/kg) under deep anesthesia. For each monkey, we harvested
448 and subjected one transplanted site for histological analysis and harvested two
449 transplanted sites and combined them for scRNA-seq analysis.

450 As a positive control for immune reactions, we created osteochondral defects (1.5
451 mm depth) in three monkeys. We transplanted 1146A1 cyiPSC-derived cyiPS-Cart into
452 one monkey and 1231C1-G cyiPSC-derived cyiPS-Cart into two monkeys. We sacrificed
453 the monkeys four weeks later, harvested the transplanted sites, and subjected them to
454 histological analysis.

455

456 **CT of monkey knee joints**

457 CT of the monkey knee joints was performed using a CT system (Aquilion TSX-
458 101A/NA; TOSHIBA, Japan). Three-dimensional images were constructed using an
459 image processing software (Aquilion TSX-101A/NA, TOSHIBA, Japan).

460

461 **MHC genotyping of monkeys**

462 MHC genotyping of monkeys was performed based on the MHC allele information
463 registered in the Immuno Polymorphism Database (<http://www.ebi.ac.uk/ipd/index.html>)
464 by Ina Research.³⁷

465

466 **Histological analysis**

467 Samples were fixed with 4% paraformaldehyde, decalcified with KCX (FALMA),
468 processed, and embedded in paraffin. Semi-serial sections were stained with HE or
469 safranin O. To assess the quality of the repaired tissue, the safranin-O-positive area and
470 the total area of the repaired tissue were measured by BZ-X800 analyzer software
471 (Keyence Corp., Japan), and the former was divided by the latter.

472 The safranin-O-positive area and the total area of articular cartilage regions
473 surrounding chondral defects were measured to assess the quality of articular cartilage
474 surrounding chondral defects; the former was divided by the latter.

475 Two individual assessors reviewed the sections in a blinded manner and scored them
476 according to a modified Wakitani histological scoring system^{40,41}. The maximum score
477 of this system is 11, and a lower score indicates repair more similar to the native articular
478 cartilage (Supplementary Table 2).⁴⁰

479 Semi-serial sections were immunostained using specific antibodies. Supplementary
480 Table 3 lists the antibodies used in this study. Anti-type I collagen, anti-type II collagen,
481 anti-GFP, and anti-PRG4 antibodies were detected using a CSA II Biotin-free Tyramide
482 Signal Amplification System Kit (Agilent Technologies, Santa Clara, CA, USA) and
483 DAB was used as the chromogen. For the anti-CD3 antibodies, immune complexes were
484 detected using secondary antibodies conjugated to Alexa Fluor 488. The antigens were
485 unmasked by treatment with hyaluronidase and EDTA.

486 The number of positively stained cells with anti-CD3 antibodies in the region below

487 the osteochondral junction was counted in four fields per knee.

488

489 **Preparation of single cells for scRNA-seq analysis**

490 The cyiPS-Cart (pre-transplant cyiPS-Cart), intact articular cartilage (cyAC),
491 fibrous tissue formed in chondral defects in the empty group (cyFT), and cyiPS-Cart in
492 chondral defects in the transplantation group (post-transplant cyiPS-Cart) were minced
493 into 1-2 mm pieces. Next, we digested these pieces with Liberase solution (for cyAC, and
494 post-transplant cyiPS-Cart: RPMI-1640 (Nacalai Tesque) supplemented with 0.2% FBS,
495 10 mM HEPES pH 7.2-7.4, 0.2-0.4 mg/mL Liberase TM (Roche), and 2 kU/mL DNase I
496 (Merck); for pre-transplant cyiPS-Cart: DMEM with 1% FBS, 1% ITS-X, 50 µg/mL
497 ascorbic acid, 1 mM sodium pyruvate, 1% nonessential amino acids, 1% penicillin-
498 streptomycin, 10 ng/mL TGF-β1, 10 ng/mL GDF5, 10 ng/mL BMP2, 0.2 mg/mL Liberase
499 TM (Roche), and 2 kU/mL DNase I (Merck)) at 37 °C, 5% CO₂ for 120–210 minutes with
500 continuous shaking. The cells were mixed 10 times using a 1,000 µL blue tip-fitted pipette
501 and then passed through a cell strainer (70 µm pore size; BD Biosciences), centrifuged at
502 4 °C for 300 × g for 5 min, and the supernatant was discarded. The cells were resuspended
503 in RPMI-1640 medium supplemented with 0.2% FBS and 10 mM HEPES.

504 For cell hashing, we biotinylated cell surface proteins using EZ-Link Sulfo-NHS-
505 Biotin (Thermo Scientific), and then stained them with 0.6 µg/mL Totalseq (A0951-
506 A0955, and A0436 (BioLegend))⁴². We selected live cells stained with A0951-A0955
507 using a FACS Aria II flow cytometer (BD Biosciences) and suspended them in a sample
508 buffer (BD Biosciences).

509

510 **cDNA synthesis using BD Rhapsody system**

511 We subjected the obtained single-cell suspensions to a BD Rhapsody system using
512 the BD Rhapsody Targeted & Abseq Reagent kit (BD Biosciences). After reverse
513 transcription, the BD Rhapsody beads were treated with exonuclease I at 37 °C for 60
514 minutes and 1,200 rpm on a Thermomixer C with a Thermotop. The resultant beads were
515 immediately chilled on ice. The supernatant was removed, and the beads were washed
516 with 1 mL WTA wash buffer (10 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM EDTA, and
517 0.02% Tween-20) and 200 µL BD Rhapsody lysis buffer (for inactivation of enzyme),

once again with 1 mL WTA wash buffer alone, twice with 500 μ L WTA wash buffer, and finally resuspended in 200 μ L bead resuspension buffer and stored at 4 °C. During the washing step, bead-containing DNA LoBind tubes were replaced twice.

521

522 **Generation of TAS-Seq library**

523 TAS-Seq libraries were generated by Immunogeneteqs Inc. (Noda City, Chiba,
524 Japan), as described previously⁴³. Briefly, reverse-transcribed exonuclease I-treated BD
525 Rhapsody beads were subjected to a terminator-assisted TdT reaction, second-strand
526 synthesis reaction, and a first/second round of whole-transcriptome amplification (WTA)
527 or Totalseq library amplification reaction. The size distribution and concentration of the
528 amplified cDNA and hashtag libraries were analyzed using a MultiNA system (Shimadzu).
529 Illumina libraries were constructed from amplified cDNA libraries (100 ng) using the
530 NEBNext Ultra II FS Library Prep kit for Illumina (New England Biolabs). Illumina
531 adapters and unique-dual barcodes were added to the hashtag libraries using PCR. The
532 size distribution and concentration of the amplified Illumina libraries were analyzed using
533 the MultiNA system and KAPA library quantification kit (KAPA Biosystems).
534 Sequencing was performed using an Illumina Novaseq 6000 sequencer (Illumina, San
535 Diego, CA, USA) and a Novaseq 6000 S4 reagent kit v1.0 or v1.5, according to the
536 manufacturer's instructions (read 1 (cell barcode): 67 bp and read 2 (cDNA): 140 (v1.0)
537 / 155 (1.5) base-pair with 8 base-pair $\times$ 2 unique-dual indexes). The pooled library
538 concentration was adjusted to 1.75 nM (v1.0) or 2.0 nM (v1.5), and the library was spiked
539 with 12% PhiX control library v3 (Illumina).

540

541 **Fastq data preprocessing and generation of the single-cell gene expression matrix**

542 To obtain the gene expression count matrix and Totalseq expression matrix for
543 every cell, fastq files of the TAS-Seq data were processed by Immunogeneteqs Inc. as
544 described previously⁴³. Bowtie2-indexes built from reference RNA sequences (cDNA and
545 ncRNA FASTA files from the Ensembl database (*Macaca_fascicularis*_6.0)⁴⁴) were used
546 to assign cDNA reads to each transcript. Associated Totalseq streptavidin/anti-biotin
547 reads were mapped to known barcode sequences (provided by BioLegend) using bowtie2-
548 2.4.2, with the following parameters: -p 2 -D 20 -R 3 -N 0 -L 8 -i S,1,0.75 -norc -seed

549 656565-reorder-trim-to3:39 -score-min L,-9,0 -mp 3,3 -np 3 -rdg 3,3. The inflection point
550 of the knee plot (total read count versus the rank of the read count) was detected using
551 the DropletUtils package⁴⁵ in R 3.6.3 (<https://cran.r-project.org/>) from the resulting
552 single-cell gene expression matrix files. Cells for which the total read count exceeded the
553 inflection point were considered valid. Demultiplexing of single cells by expression of
554 Totalseq streptavidin/anti-biotin was performed as described previously⁴⁶.

555

556 **Background subtraction of TAS-Seq expression matrix by distribution-based error** 557 **correction**

558 To reduce the background read counts of each gene possibly derived from RNA
559 diffusion during the cell lysis step within the BD Rhapsody cartridge and reverse
560 transcription, a distribution-based error correction was included in the BD Rhapsody
561 targeted scRNA-seq workflow performed by Immunogeneteqs Inc., as previously
562 reported⁴⁶. Briefly, the genes for which the $\log_2(x+1)$ -transformed maximum expression
563 was over 8 were selected, and a biexponential transformation was applied to each gene
564 count using the FlowTrans package⁴⁷ in R 3.6.3. Next, Gaussian mixture components
565 were detected using the mclust package⁴⁸ in R 3.6.3. The average expression of each
566 component was calculated, and the genes for which the maximum average expression of
567 each component was over 5.5 were selected. If the difference between the average
568 expression of each component and its maximum expression was greater than 5, the
569 expression level of the components was considered as background gene expression, and
570 the converted expression of the components was set to 0.

571

572 **Single-cell clustering and annotation**

573 We clustered single cells from each dataset using Seurat v 4.0.3⁴⁹ in R 4.1.0. The
574 Seurat object for each dataset was created using the CreateSeuratObject function (min.
575 cells =5, min. genes =500). Cells for which the percentage of mitochondrial genes was
576 greater than the threshold were filtered using the subset function in Seurat v4.0. The
577 expression data were normalized using the NormalizeData function (scale factor =
578 1,000,000, according to the analytical parameter used by Muris⁵⁰). Cells were categorized
579 into the S, G1, or G2/M phases by scoring cell cycle-associated gene expression using the

CellCycleScoring function. Highly variable genes in each dataset were identified using the FindVariableFeatures function with the following parameters: selection.method = "vst," nfeatures = 5,000, mean.cutoff = c(0.1, Inf), and dispersion.cutoff = c(0.5, Inf). The expression data were scaled using the ScaleData function. The read counts of each cell within each dataset were regressed as confounding factors in the ScaleData function. Principal component analysis (PCA) was performed using the RunPCA function, and the top 42 PCs were selected for dimensional reduction using UMAP. We determined the cluster resolution of the values. DEGs of each cluster were determined using the FindMarkers function with a 0.05 p_val_adj threshold between the two groups (i.e., cell clusters). DEGs were further analyzed by IPA v01-20-04 (QIAGEN) to identify upstream genes and pathways.

591

592 RNA velocity analysis

We performed the RNA velocity analysis as described previously⁵¹. TAS-Seq data cDNA reads were mapped to the reference genome (*Macaca fascicularis*_6.0 for *macaca fascicularis* data, and GRCh38 release-101 for human data) using HISAT2-2.2.1⁵² and the following parameters were used: -q -p 6 -rna-strandness F -very-sensitive -seed 656565 -reorder -omit-sec-seq -mm. For the HISAT2 index build, a corresponding ensembl gtf file was filtered to retain protein-coding RNA, long non-coding RNA, and T cell chain/immunoglobulin chain annotations according to the 10X Genomics's method (<https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/advanced/references#mkgtf>). Then, the cell barcode information of each read was added to the HISAT2-mapped BAM files, and associated gene annotations were assigned using featureCounts v2.0.2⁵³ with the following parameters: -T 2 -Q 0 -s 1 -t gene -g gene_name -primary -M -O -largestOverlap -fraction -R BAM. In the featureCounts analysis, a "gene" annotation was used to capture unspliced RNA information for the RNA velocity analysis, and primary annotations were kept. The resulting BAM file was split using valid cell barcodes and nim 1.0.6 and hts-nim v0.2.23, the split files were processed into loom files using velocityto run (version 0.17.17) with the -c and -U options, and the loom files were concatenated using the loompy.combine function (version 3.0.6)⁵⁴. Then, we used scVelo⁵⁵ for RNA velocity

analysis. The loom files were read to an AnnData object. After estimating the RNA velocity, we inferred the trajectory using PAGA⁵⁶. The velocity-inferred directionality extended the trajectory.

Isolation and culture of cells from pre-transplant cyiPS-Cart

The pre-transplant cyiPS-Cart was treated with 0.25% trypsin-EDTA (Thermo Fisher Scientific) for 1 h and subsequently treated overnight with 4 mg/mL collagenase D (Roche) in DMEM supplemented with 1% PC/SM. After washing, cells were suspended in DMEM supplemented with 10% FBS or chondrogenic medium.

A total of 3.0×10^5 cells from pre-transplant cyiP-Cart were plated in a ϕ 35 mm dish and cultured in DMEM supplemented with 10% FBS in the presence or absence of 100 ng/mL TGF- β 1 (Peprotech). A total of 2.3×10^5 cells from pre-transplant cyiPS-Cart were cultured in chondrogenic medium in the presence or absence of 100 μ M TGF- β inhibitor SB431542 (Cayman). The cells were collected 48 h later for mRNA expression analysis.

Conventional and conditional *Sik3* knockout mice and *Sik3* transgenic mice

All mice used were C57BL/6. *Sik3*^{-/-} mice²⁵ and *Sik3*^{lox/lox} mice²⁴ have been previously described. *11Enh-Cre* mice are transgenic mice expressing Cre under the control of *Col11a2* promoter/enhancer sequences⁵⁷. *11Enh-Cre* transgenic mice and *Sik3*^{lox/lox} mice were mated to generate *Sik3* conditional knockout mice, in which *Sik3* was specifically deficient in chondrocytes. *Sik3*^{-/-} and *11Enh-Cre*; *Sik3*^{lox/lox} mice exhibited similar cartilage phenotypes. *Col11a2-hSIK3* transgenic mice overexpressing human SIK3 in chondrocytes under the *Col11a2* promoter/enhancer control were described previously²⁵.

Culture of mouse primary chondrocytes

Primary chondrocytes were prepared from *Sik3*^{-/-} knockout mice at 18.5 postcoitus and *Col11a2-hSIK3* transgenic mice 3 days after birth, as described previously⁵⁸. Briefly, epiphyseal cartilage was dissected from the knee, elbow, shoulder joints, and femoral

heads of mice and digested with 3 mg/mL collagenase D (Roche) in DMEM/F12 (Invitrogen) containing 5% FBS and 1% penicillin-streptomycin (Life Technologies) at 37 °C overnight. Approximately 5×10^5 primary chondrocytes were obtained from each mouse and cryopreserved in LaboBanker (Kurabo Industries Ltd.). Before the experiments, the cells were lysed, plated, and cultured in DMEM/F12 supplemented with 5% FBS, and 1% penicillin-streptomycin for less than 10 days.

Chondrocytes were seeded (0.5×10^5 cells/well) into 12-well tissue culture plates (Corning). Chondrocytes were cultured in DMEM/F-12 containing 5% FBS and 1% penicillin-streptomycin (Invitrogen) at 5% CO₂ in humidified air. Before experiments, cells were pretreated overnight with a starvation medium (serum-free). For experiments, cells were treated with 1, 5 or 10 ng/mL TGF- β 1 (peprotech) or 10 or 100 μ M forskolin (F3917, Sigma-Aldrich) (in DMSO) for 30 min or 1 h for immunoblot analysis and 6 h for mRNA expression analysis.

Application of FFSS

We followed a previously described method²². Mouse primary chondrocytes were seeded (1×10^5 cells/well) in 6-well tissue culture plates (diameter = 3.48 cm) (Corning). Chondrocytes were cultured in DMEM/F-12 containing 5% FBS and 1% penicillin-streptomycin (Invitrogen) with 5% CO₂ in humidified air for 2-24 h. Thirty minutes before exposure to static or FFSS conditions, the medium was replaced gently with 4 mL fresh culture medium. For cultures exposed to FFSS, culture dishes were placed on an orbital shaker (0.5-cm radius of gyration) (IKA, KS 260 basic) set to rotate at 300 rpm at 37 °C, which exposed the chondrocytes to a steady laminar, non-pulsatile shear stress of approximately 7.6 dyn/cm².

Immunoblot analysis

Mouse primary chondrocytes were lysed in RIPA buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM EDTA, 1% NP-40, complete protease inhibitors from Roche, and phosphatase inhibitor cocktail 1 and 2 from Sigma-Aldrich) and subjected to SDS-PAGE.

671 The separated proteins were electroblotted and the membranes were
672 immunostained with rabbit anti-SIK3 (Abcam, 1:500), rabbit anti-pSIK3 (pT411)
673 (KINEXUS, 1:1,000), rabbit anti-Smad2/3 (CST #8685S, 1:1000, rabbit anti-pSmad3
674 (CST #9520S, 1:1000), and rabbit anti- β -actin (Cell Signaling, 1:1,000) antibodies. Goat
675 anti-mouse IgG-HRP (Santa Cruz, 1:5,000) or goat anti-rabbit IgG-HRP (Santa Cruz, 1:
676 5,000) were used as secondary antibodies. ECL system and LAS4000 (GE Healthcare)
677 were used for chemiluminescent immunodetection. pSIK3(pT411) levels were quantified
678 relative to β -actin levels by using the FUSION FX software (Vilber, France). The
679 uncropped blots are provided in Source Data and Supplementary Fig. 9.

680

681 **mRNA expression analysis**

682 Total RNAs were extracted using RNeasy (Qiagen). For quantitative reverse
683 transcription PCR (RT-PCR), total RNA was reverse-transcribed into first-strand cDNA
684 using ReverTra Ace (Toyobo) and an oligo(dT)20 primer. PCR amplification was
685 performed using the KAPA SYBR FAST qPCR Master Mix ABI prism kit (KAPA
686 Biosystems, Wilmington, USA) and StepOnePlus Real-Time PCR System (Thermo
687 Fisher Scientific). The sequences of the PCR primers used are listed in Supplementary
688 Table 4. The RNA expression levels of target genes were normalized to that of GAPDH
689 or β -actin mRNA expression, and the results indicate the relative expression of the
690 molecules.

691

692 **Statistical analysis**

693 The data are shown as mean $\pm$ standard error (SE). We performed two-tailed Student's
694 *t*-test for parametric data and one-way analysis of variance (ANOVA) with Tukey's HSD
695 test for multiple comparisons. Statistical significance was set at $P < 0.05$.

696

697

698 **Data availability**

699 All the original data are available upon request from the authors. The scRNA-seq datasets
700 have been deposited in the GEO database under accession code GSE206120

701 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE206120]. Source data are
702 provided with this paper.

703

704 **Code Availability**

705 The code used in this study is provided as Supplementary Code 1.

706

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878 **Acknowledgments**

879 We thank Chieko Matsuda, Masumi Sanada, Hiroki Hagizawa, and Yuya Nishijima
880 for their assistance and helpful discussion. We thank the iPS Cell Research Fund for
881 research support. This study was supported by JSPS KAKENHI Grant No. 18H02923 (to
882 N.T.) and WPI Premium Research Institute for Human Metaverse Medicine (PRIME) (to
883 N.T.) from the Japan Society for the Promotion of Science. This study was also supported
884 by the Center for Clinical Application Research on Specific Disease/Organ (type B) Grant
885 No. 21bm0304004h0009 (to N.T.); Research Project for Practical Applications of
886 Regenerative Medicine Grant No. 21bk0104079h0003 (to N. T.); Practical Research
887 Project for Rare/Intractable Diseases (step 1) Grant No. 21ek0109452h0002 (to N.T.);
888 Core Center for iPS Cell Research Grant No. 20bm0104001h0008 (to N.T.); and the
889 Acceleration Program for Intractable Diseases Research utilizing disease-specific iPS
890 cells Grant No. 20bm0804006h0004 (to N.T.) from the Japan Agency for Medical
891 Research and Development (AMED).

892

893 **Author contributions**

894 K.A., S.M., and N.T. designed experiments. K.O. prepared cyiPSCs. A.Y. created the
895 cyiPS-cart. K.A. transplanted cyiPS-Carts into monkeys. K.A. performed CT and
896 histological analyses. K.A, M.M., S.K., and N.T. performed scRNA-seq analysis. N.H.
897 and Y.T. performed experiments regarding Sik3. K.A. and N.T. wrote the manuscript.

898

899 **Competing interests:**

900 N.T. is an inventor and Kyoto University is a holder of the patent on “An efficient
901 chondrocyte induction method” (PCT/JP2014/079117). This patent is licensed to Asahi
902 KASEI corporation. Y.T. is an employee of Asahi KASEI. The remaining authors declare
903 no competing interests.

904

905 **Figure legends**

906 **Figure 1.** Transplantation of cyiPS-Cart in the knee joints in a primate model.

- 907 a) Two categories of articular cartilage defect. *Left*: chondral defects extending down to
908 but not through the subchondral bone. *Right*: osteochondral defects extending down
909 through the subchondral bone.
- 910 b) Primate model for cyiPS-Cart transplantation. Chondral defects were created in the
911 femoral trochlear ridge of the right knee joints in *cynomolgus monkeys*. CyiPS-Cart
912 (transplantation group) or nothing (empty group) were transplanted into the defects.
913 The monkey image is taken from [[https://www.flaticon.com/free-](https://www.flaticon.com/free-icon/monkey_47138)
914 [icon/monkey_47138](https://www.flaticon.com/free-icon/monkey_47138)] following the Flaticon license guidelines.
- 915 c) Gross appearance of the joint surface 4 weeks (*left*) and 17 weeks (*right*) after surgery.
916 Data are representative of three monkeys.

917

918 **Figure 2.** Analysis of immune reactions following allogeneic transplantation of cyiPS-
919 Cart into chondral defects in the knee joints four weeks after transplantation.

- 920 a) Semi-serial histological sections were stained with safranin O or HE, or
921 immunostained for CD3. Scale bars, 100 μ m.
- 922 b) The number of CD3⁺ cells per microscopic field was determined. Four fields were
923 used for each monkey. Three monkeys were used in each group. Each mark indicates
924 one field, and different shapes of marks indicate different monkeys. Error bars denote
925 mean $\pm$ SE. **** $P < 0.0001$ by one-way ANOVA with post-hoc Tukey HSD test (n
926 = 12 fields). Source data are provided as a Source Data file.

927

928 **Figure 3.** Qualities of repaired tissue in chondral defects.

- 929 a) Samples were harvested 4 or 17 weeks after transplantation. Semi-serial sections were
930 stained with Safranin O, HE, or picrosirius red. Sections stained with picrosirius red
931 were observed under a polarized microscope. A magnification of the boxed regions
932 that cover repaired tissue and native articular cartilage in the third row is indicated in
933 the bottom row. Scale bars, 100 μ m.
- 934 b) Sections stained with Safranin O were subjected to a modified Wakitani histological
935 scoring system ($n = 3$ monkeys in each group) and evaluated by two independent

936 assessors in a blinded manner. Error bars denote mean $\pm$ SE. Source data are provided
937 as a Source Data file.

938 c) Magnifications of remaining cartilage located between the bottom of the defect and
939 bone in (a). *Dotted lines* indicate bottom of defects. Safranin O staining. Scale bars,
940 100 μ m.

941

942 **Figure 4.** Immunohistochemical staining of repaired chondral defects.

943 a) Samples were harvested 4 or 17 weeks after transplantation. Semi-serial sections were
944 immunostained for GFP, type II collagen (COL2), and type I collagen (COL1). The
945 boxed regions in the second row are magnified in the third row. Data are
946 representative of three monkeys.

947 b) Semi-serial histological sections of samples at 17 weeks after transplantation were
948 immunostained for CD3. Data are representative of three monkeys.

949 Scale bars, 100 μ m.

950

951 **Figure 5.** scRNA-seq analysis and chondrogenic differentiation of cyiPSCs into pre-
952 transplant cyiPS-Cart.

953 a) Schematic representation of samples subjected to scRNA-seq analysis.
954 Undifferentiated cyiPSCs (cyiPSC), cyiPS-Cart (pre-transplant cyiPS-Cart), intact
955 articular cartilage (cyAC), fibrous tissue formed in chondral defects in the empty
956 group (cyFT), and cyiPS-Cart in chondral defects in the transplantation group (post-
957 transplant cyiPS-Cart) 17 weeks after surgery.

958 b) Ridgeplot (Seurat) showing the distribution of single-cell gene expression in each
959 sample. The x-axis of each panel represents the expression levels of the indicated
960 genes. The y axis represents the number of cells.

961 c) CyiPSCs and pre-transplant cyiPS-Cart cells were projected onto UMAP plots with a
962 parameter resolution of 0.5.

963 d) Marker gene expression levels are indicated in each cell projected on the UMAP plot
964 using the feature plot function.

965

966 **Figure 6.** scRNA-seq analysis of cyAC, cyFT, pre-transplant cyiPS-Cart, and post-
967 transplant cyiPS-Cart.

- 968 a) The VlnPlot (Seurat) shows the distribution of single-cell gene expression in each
- 969 sample. The y-axis of each panel represents the expression levels of the indicated
- 970 genes.
- 971 b) After reducing the cell number for each sample to 320, the data from the samples were
- 972 integrated. The cells were then clustered with a parameter resolution of 0.2 and
- 973 projected onto the UMAP plots.
- 974 c) UMAP plot in (b) separated by samples.
- 975 d) The ratio of the number of cells in each cell cluster in each sample (c) is plotted.
- 976 e) *COL2A1* and *COL1A1* expression levels are indicated in each cell projected on the
- 977 UMAP plot using the feature plot function.
- 978 f) Heatmap revealing the scaled expression of differentially expressed genes for each
- 979 cluster defined in (b).
- 980 g) Canonical pathways enriched for each cluster based on differentially expressed genes.
- 981 The results of Clusters #3 and #4 were omitted because there were very few cells in
- 982 these clusters.

983

984 **Figure 7.** Expression of *PRG4* in cyAC, pre-transplant cyiPS-Cart, and post-transplant

985 cyiPS-Cart.

- 986 a) The expression level of each gene in pre-transplant cyiPS-Cart is plotted on the x-axis
- 987 and the expression level in post-transplant cyiPS-Cart is plotted on the y-axis.
- 988 b) VlnPlot of *PRG4* expression for each sample.
- 989 c) *PRG4* expression levels indicated in each cell projected on the UMAP plot in Fig. 6b,
- 990 using the FeaturePlot function.
- 991 d) Histological sections were immunostained for PRG4 expression. A magnification of
- 992 the boxed regions in the top row is shown in the bottom row. Data are representative
- 993 of three cyiPS-Cart organoids and three monkeys. Scale bars, 100 μ m.
- 994 e) Cells from the pre-transplant cyiPS-Cart were cultured in the presence or absence of
- 995 TGF- β 1 (*left*) or TGF- β inhibitor, SB431542 (*right*). *PRG4* mRNA expression was
- 996 analyzed using real-time RT-PCR. Error bars denote means $\pm$ SE. $**P = 0.0048$, $**P$
- 997 $= 0.0017$ by two-tailed Student's *t*-test ($n = 3$ dishes). Data are representative of three
- 998 independent experiments. Source data are provided as a Source Data file.

999

1000 **Figure 8.** Involvement of Sik3 function and fluid flow shear stress (FFSS) on *Prg4*
1001 expression in mouse chondrocytes.

1002 a) Immunoblot expression analysis of Sik3 phosphorylated at T411, an inactive form of
1003 Sik3, in wild-type mouse primary chondrocytes treated with 10 μ M forskolin for 30
1004 min. *Left*: blots representative of three independent experiments are shown. *Right*:
1005 quantification of pSik3(pT411) to β -actin ratio in cultured chondrocytes with or
1006 without forskolin treatment. Error bars denote mean $\pm$ SE. $**P = 0.0041$ by two-tailed
1007 Student's *t*-test ($n = 3$).

1008 b) Real-time RT-PCR analysis of *Prg4* expression in wild-type mouse primary
1009 chondrocytes treated with 10 μ M forskolin for 6 h. Error bars denote mean $\pm$ SE. $*P$
1010 $= 0.0108$ by two-tailed Student's *t*-test ($n = 6$ dishes). Data are representative of three
1011 independent experiments.

1012 c) Real-time RT-PCR analysis of *Prg4* and *Col10* expression in primary chondrocytes
1013 obtained from Sik3 knockout (*Sik3*^{-/-}) and Sik3 transgenic (*Sik3*^{tg}) mice. Error bars
1014 denote mean $\pm$ SE. $****P < 0.0001$, $****P < 0.0001$, $***P = 0.0003$, $**P = 0.0012$,
1015 $n = 3$, two-tailed Student's *t*-test ($n = 3$ dishes). Data are representative of two
1016 independent experiments.

1017 d) Immunohistochemical analysis of *Prg4* expression in the knee joints of *Sik3*
1018 conditional knockout (*11Enh-Cre; Sik3*^{fllox/fllox}) mice lacking Sik3 expression in
1019 chondrocytes 14 days after birth. Yellow arrows indicate the thickness of the area in
1020 which *Prg4* was expressed. Data are representative of five conditional knockout mice
1021 and four *Sik3*^{fllox/+} mice. Scale bars: 100 μ m.

1022 e) Real-time PCR analysis of *Prg4* expression in wild-type (*Sik3*^{+/+}) and *Sik3* knockout
1023 (*Sik3*^{-/-}) primary chondrocytes subjected to FFSS for the indicated period. Data are
1024 representative of two independent experiments.

1025 Source data are provided as a Source Data file.

Figure 1

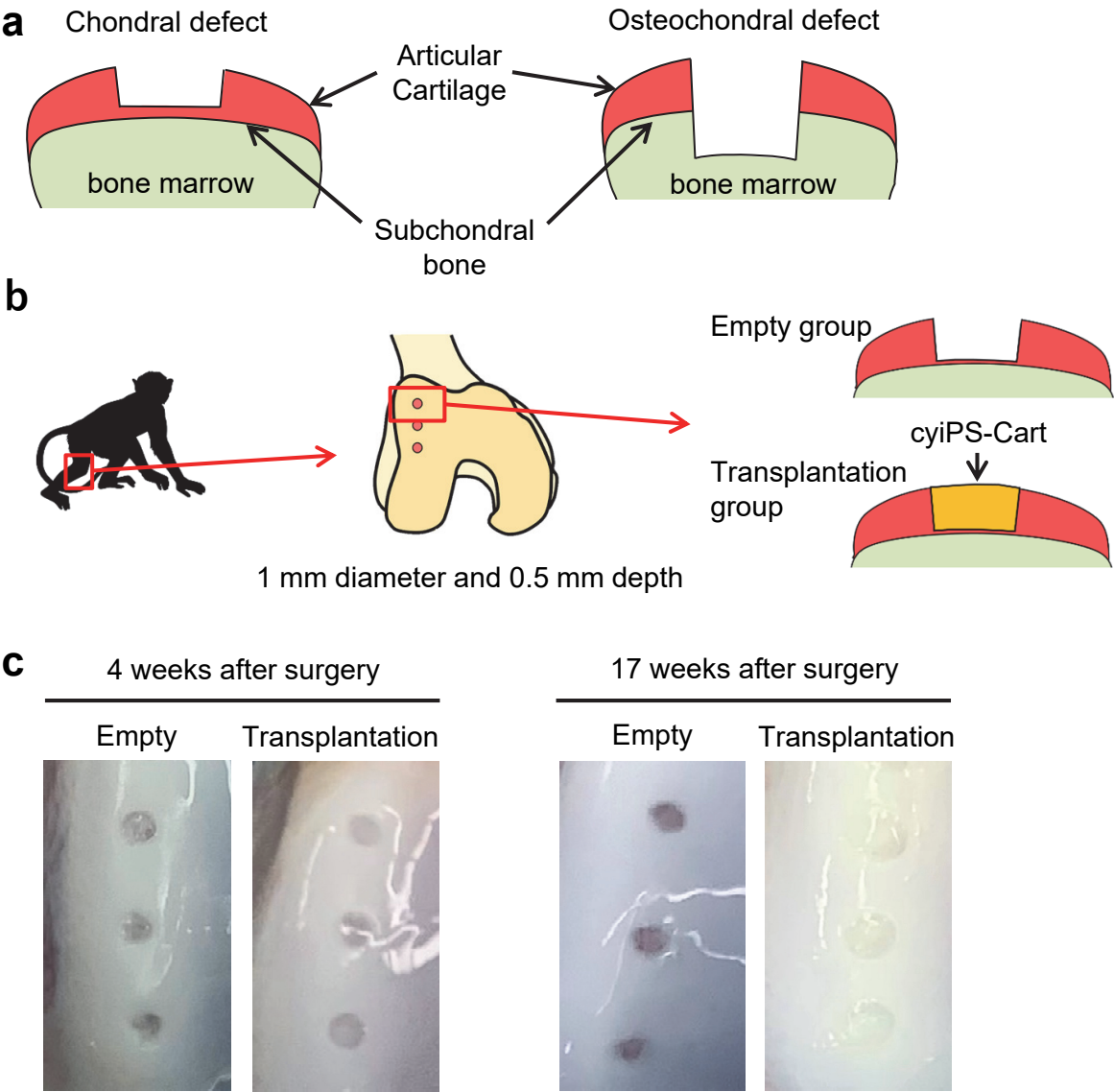


Figure 2

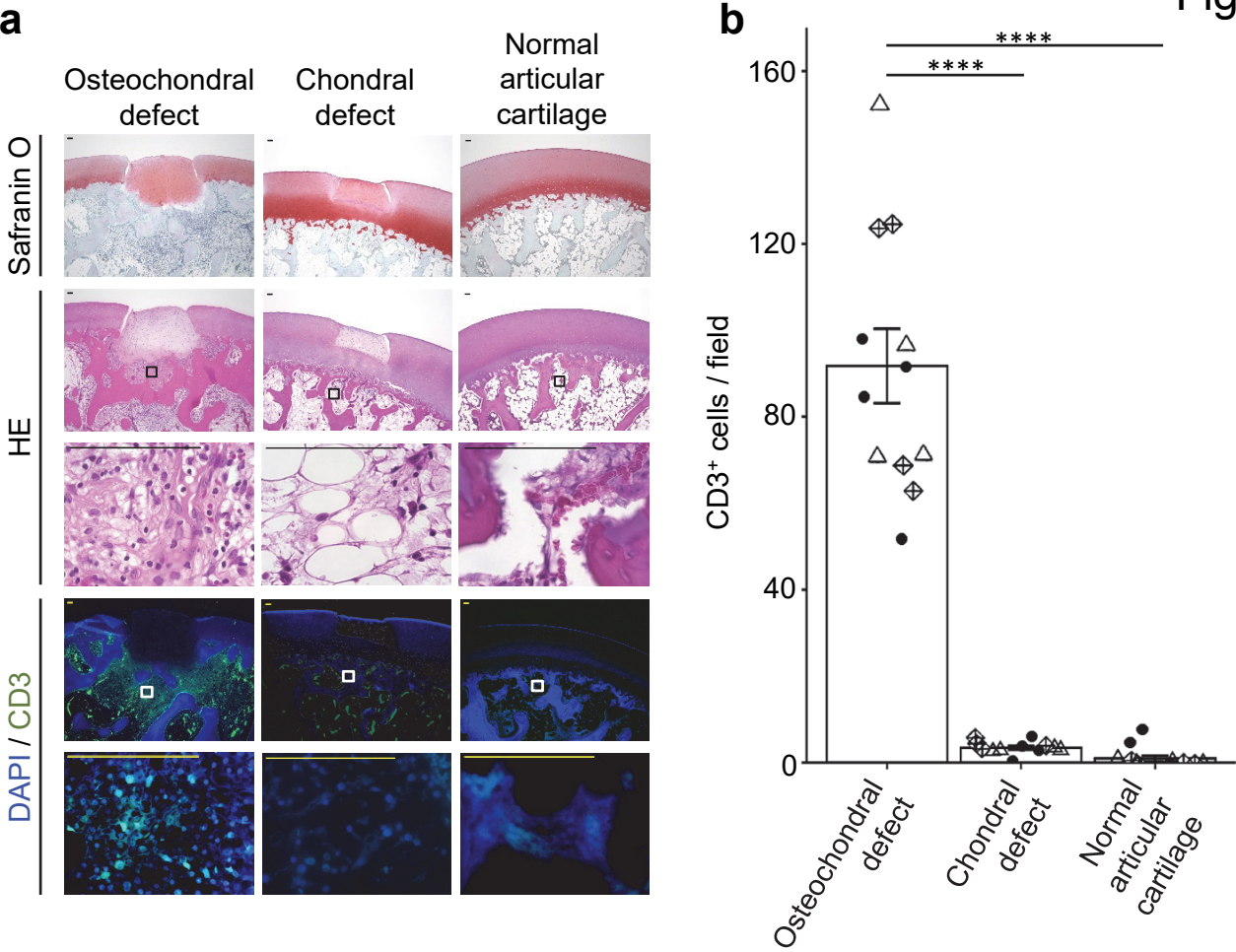
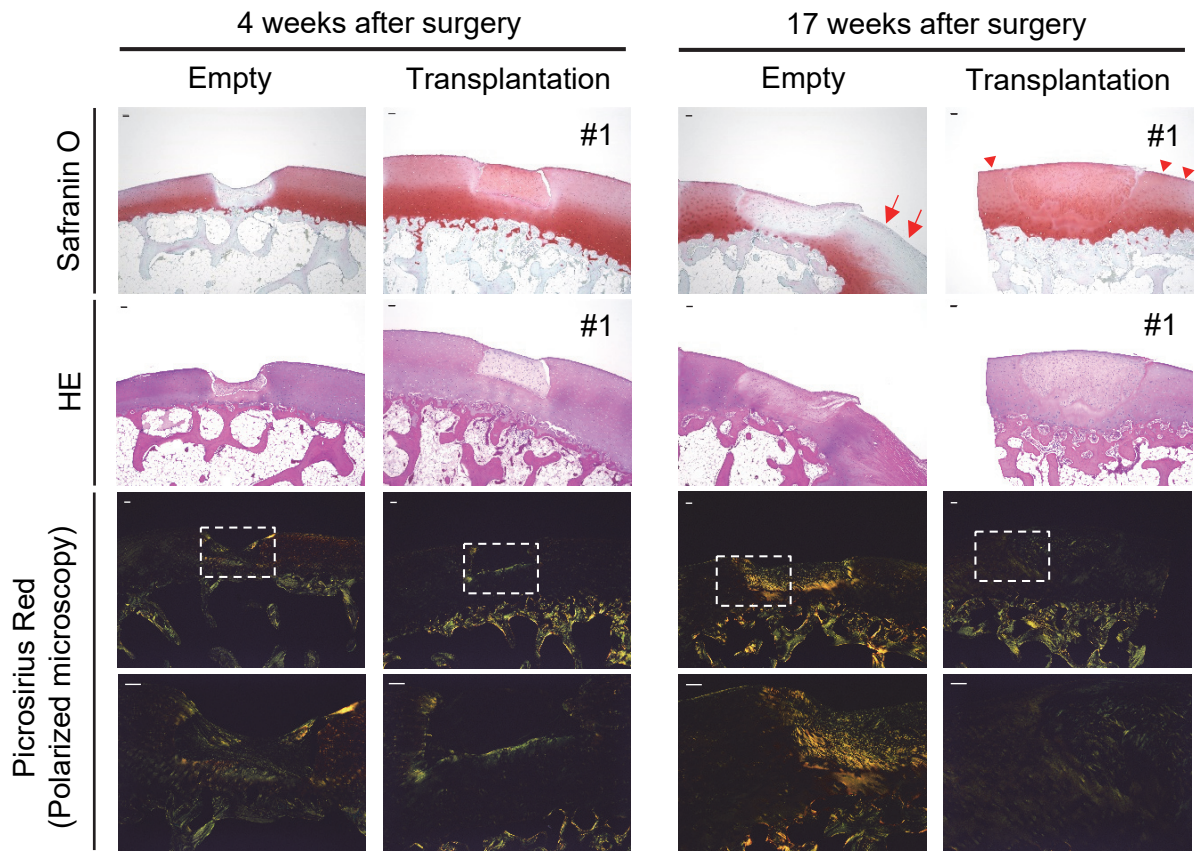
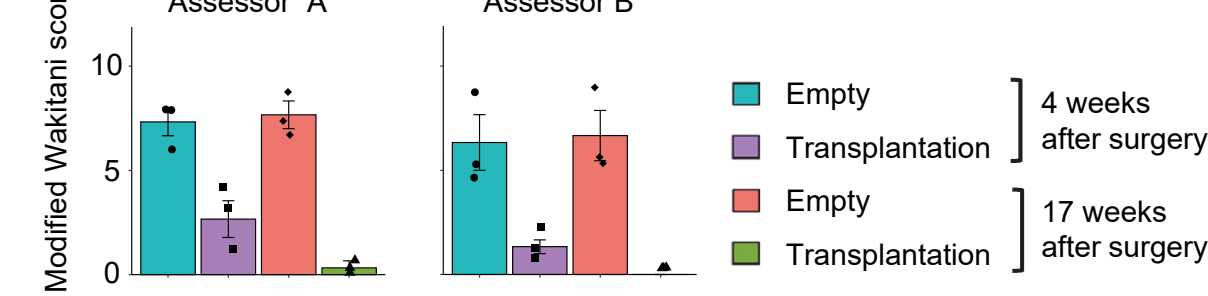


Figure 3

a



b



c

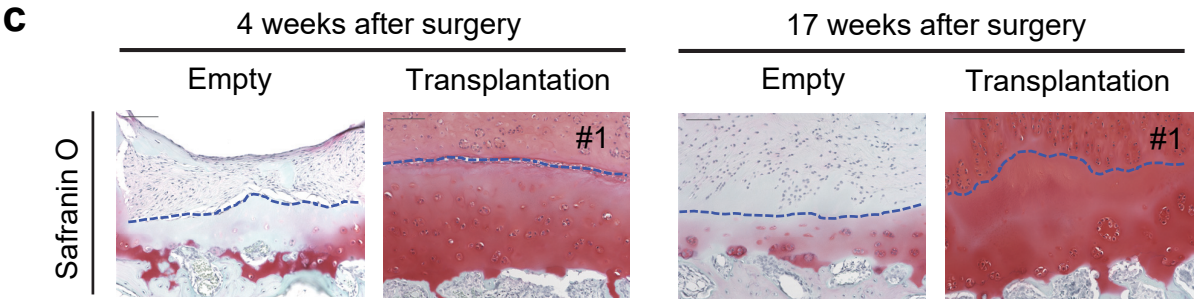


Figure 4

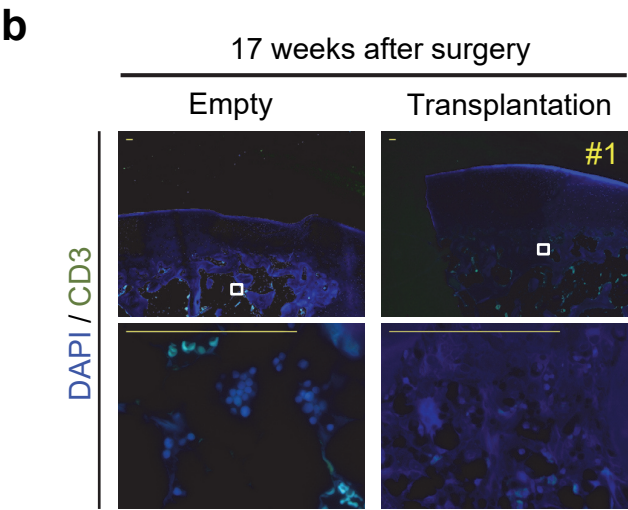
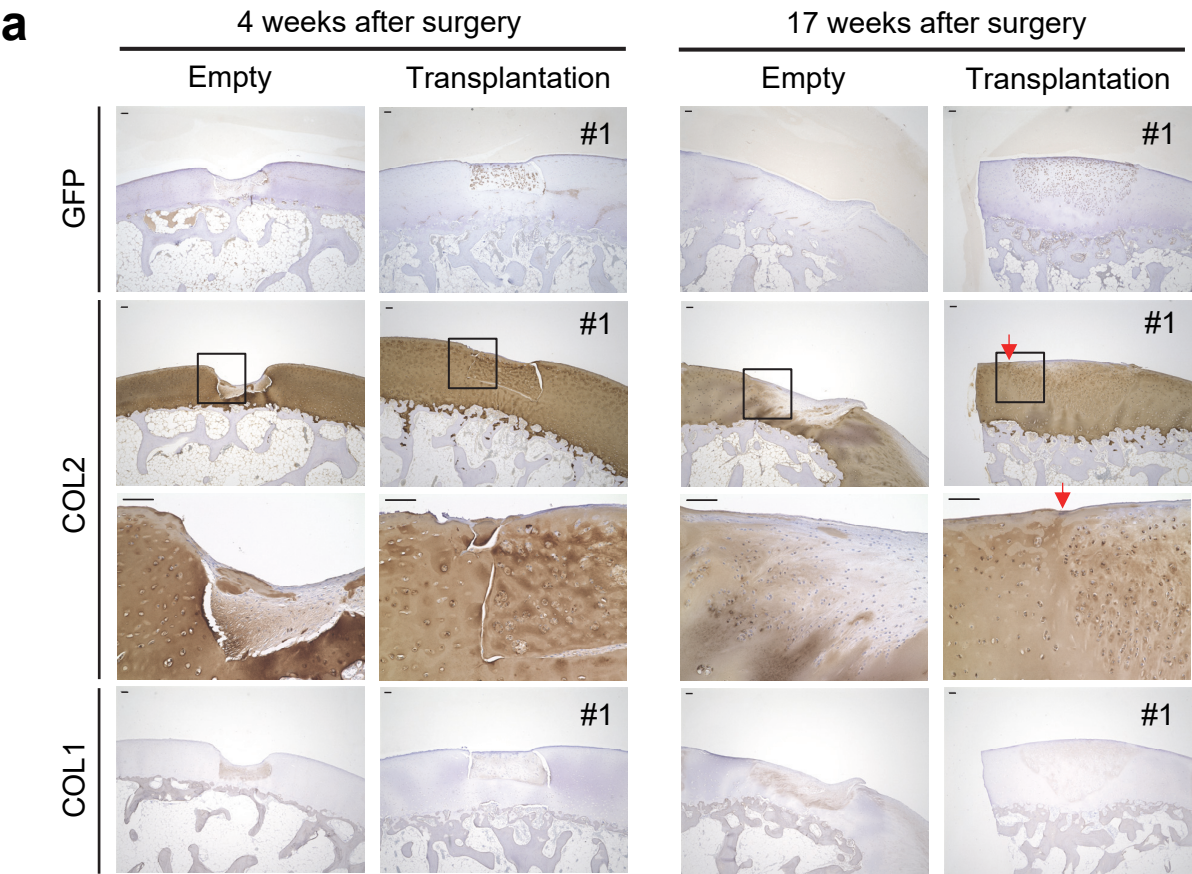


Figure 5

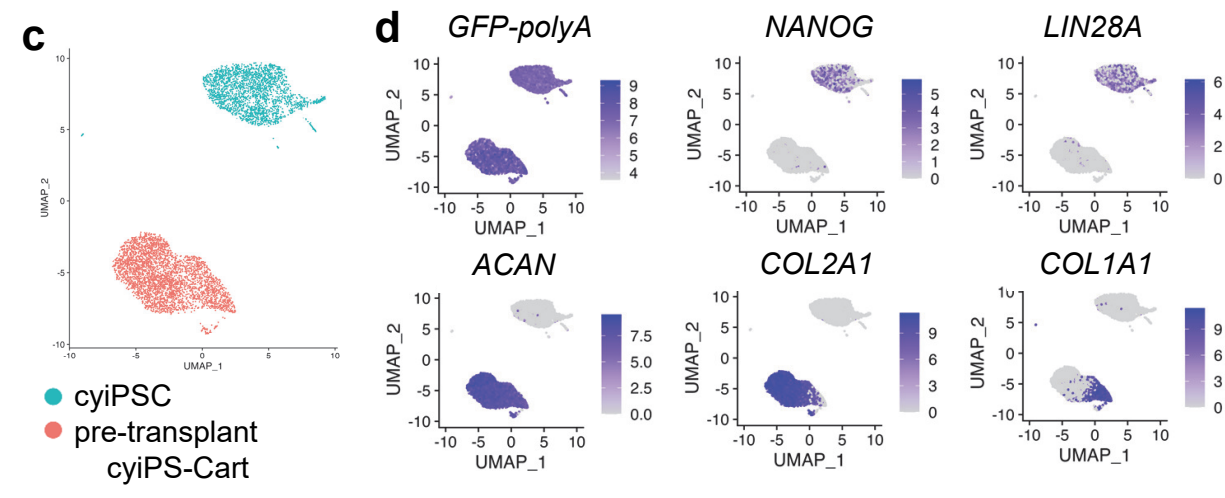
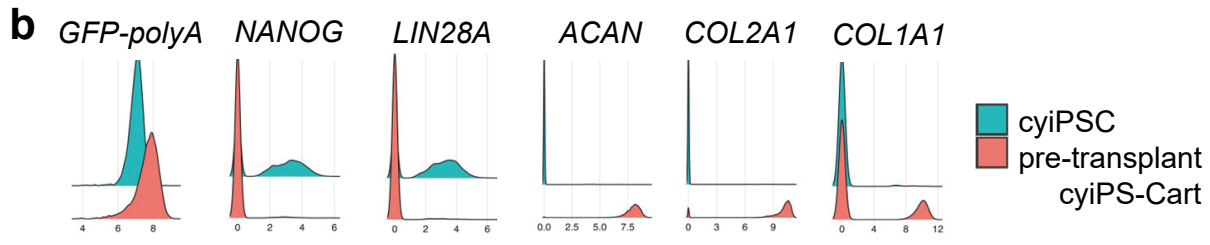
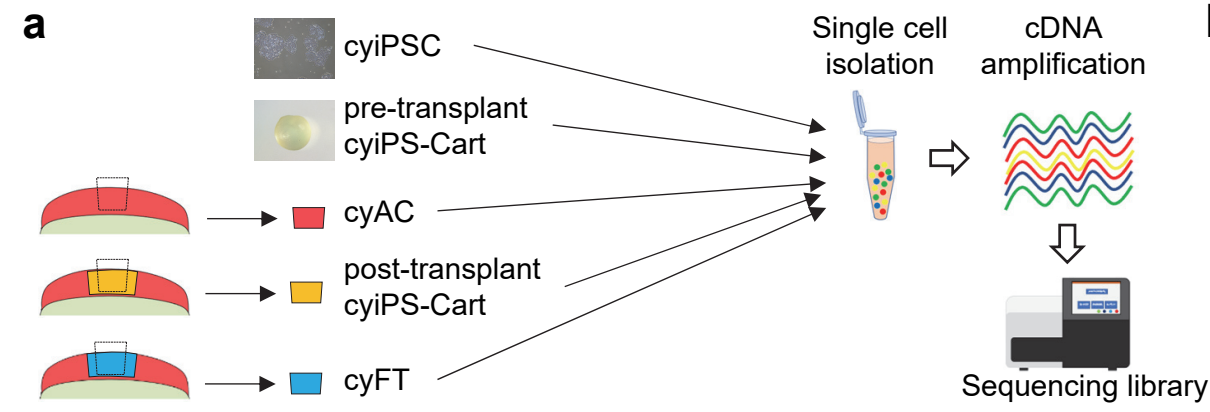


Figure 6

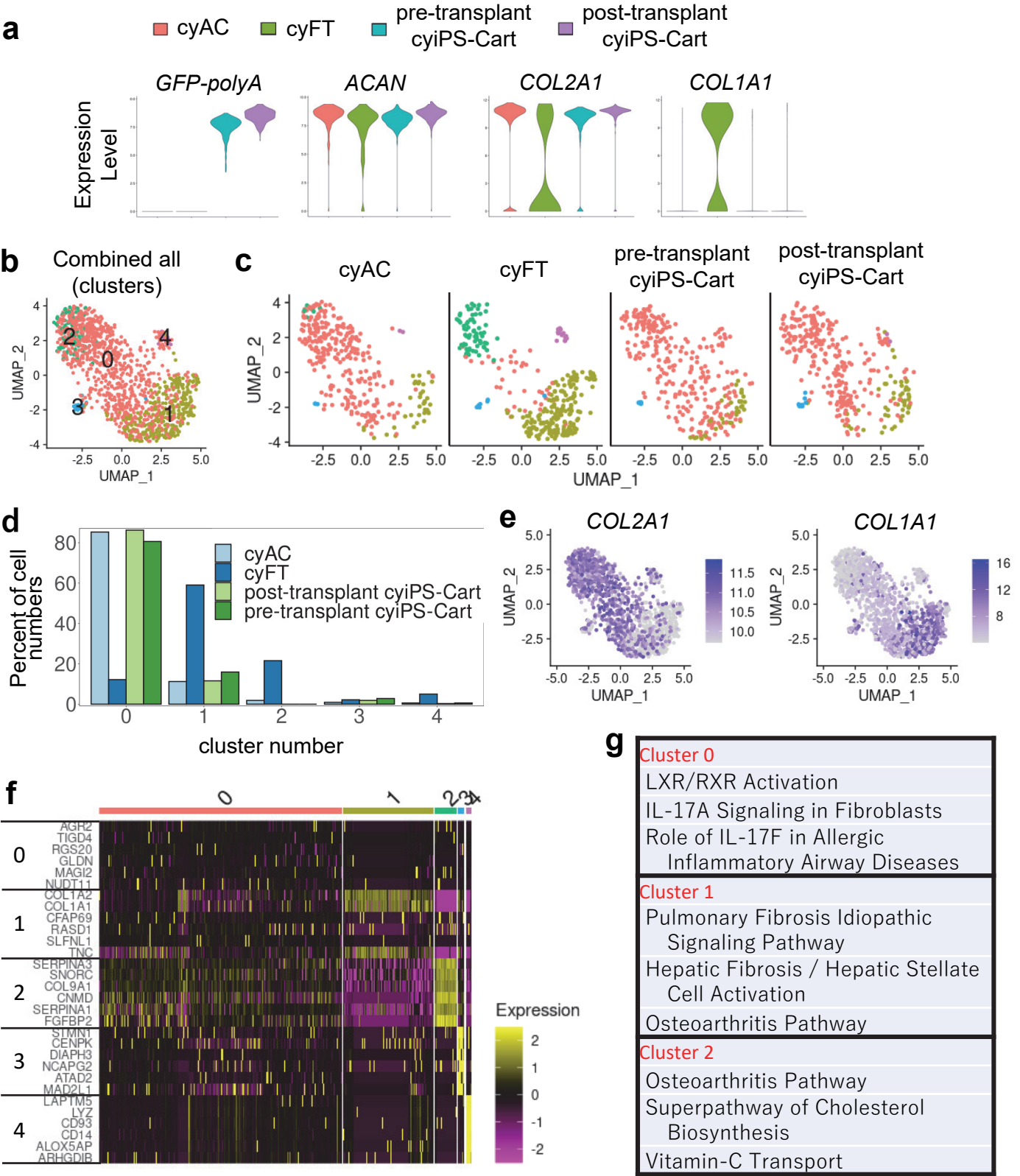


Figure 7

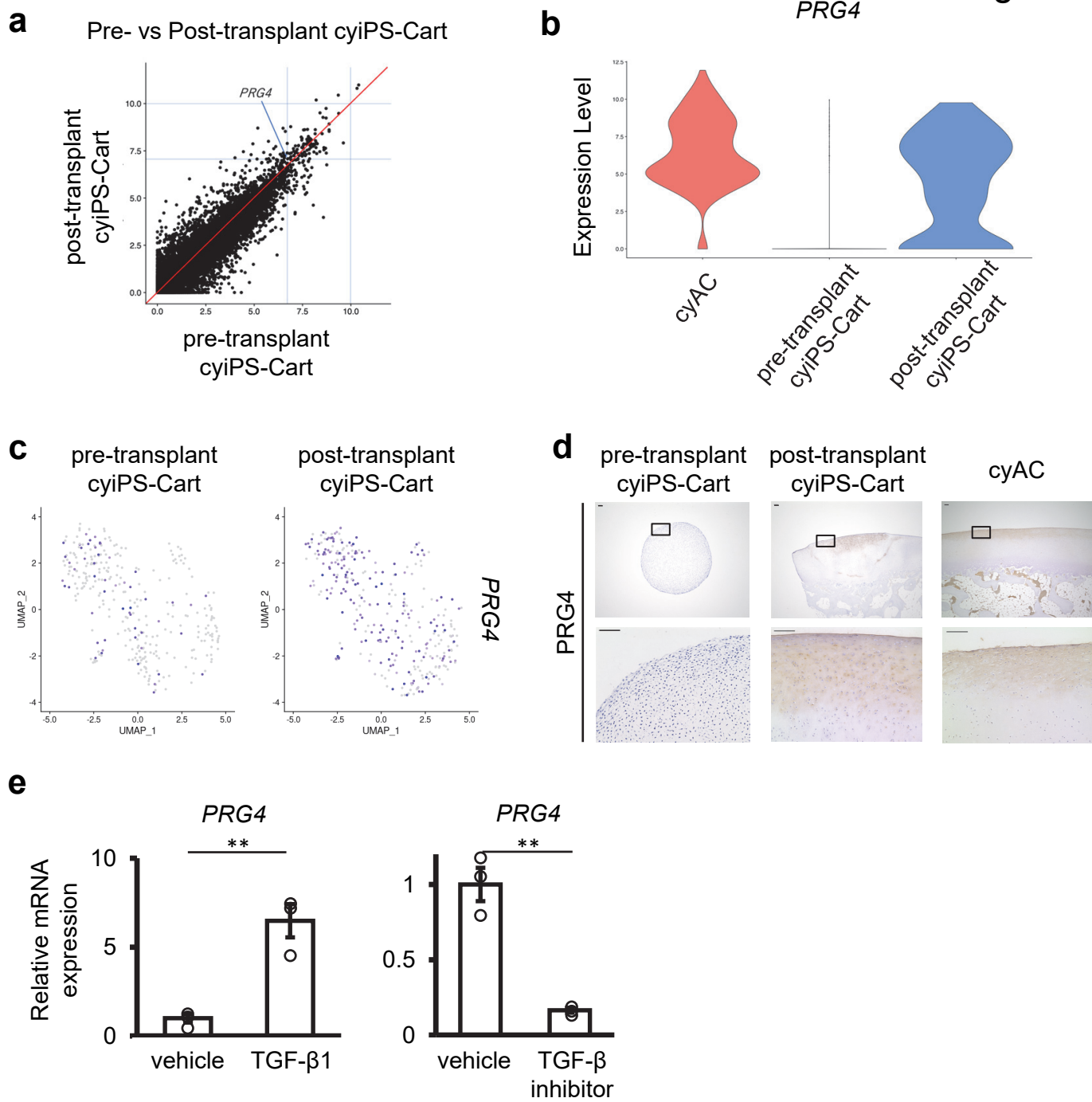
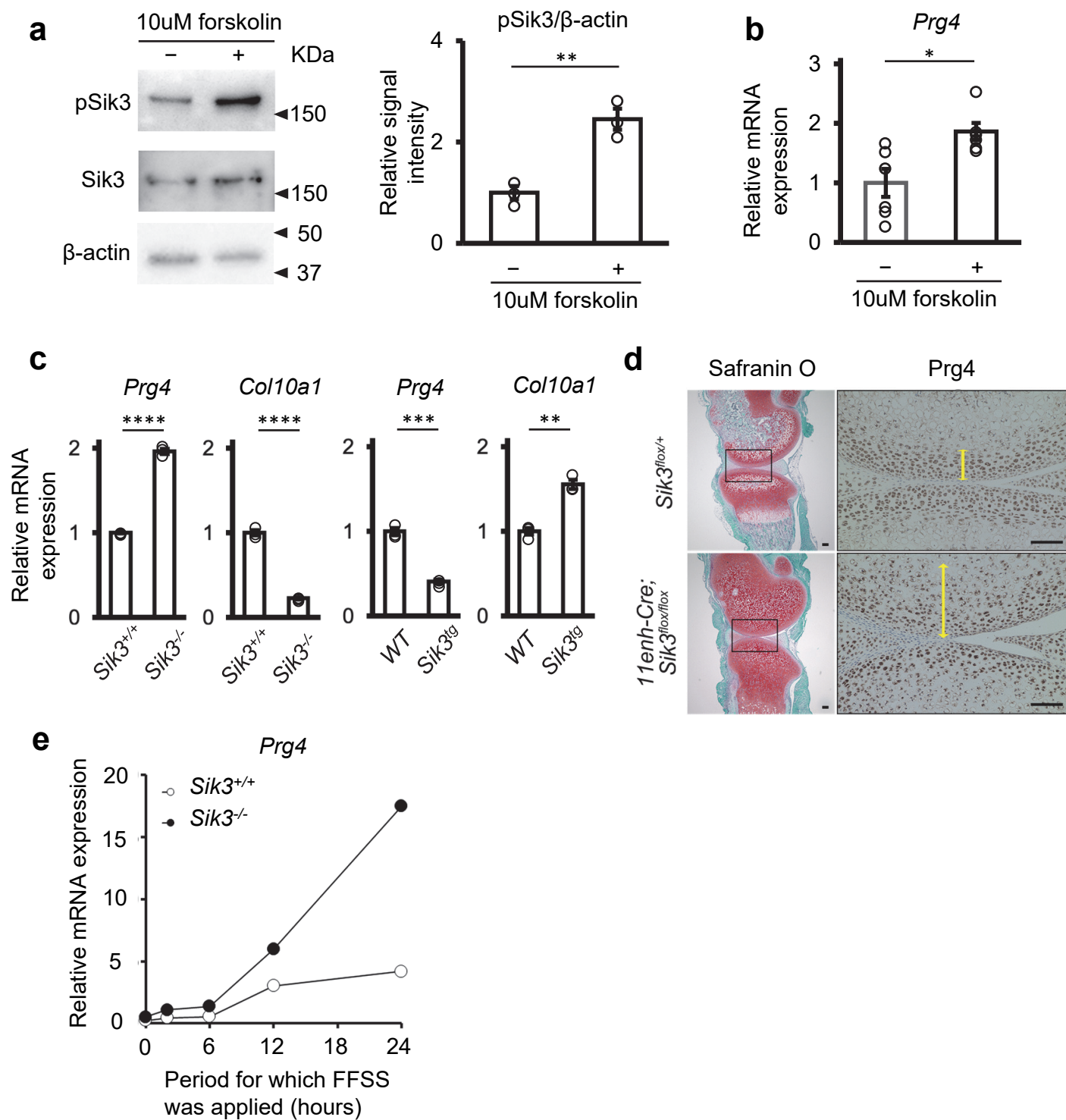


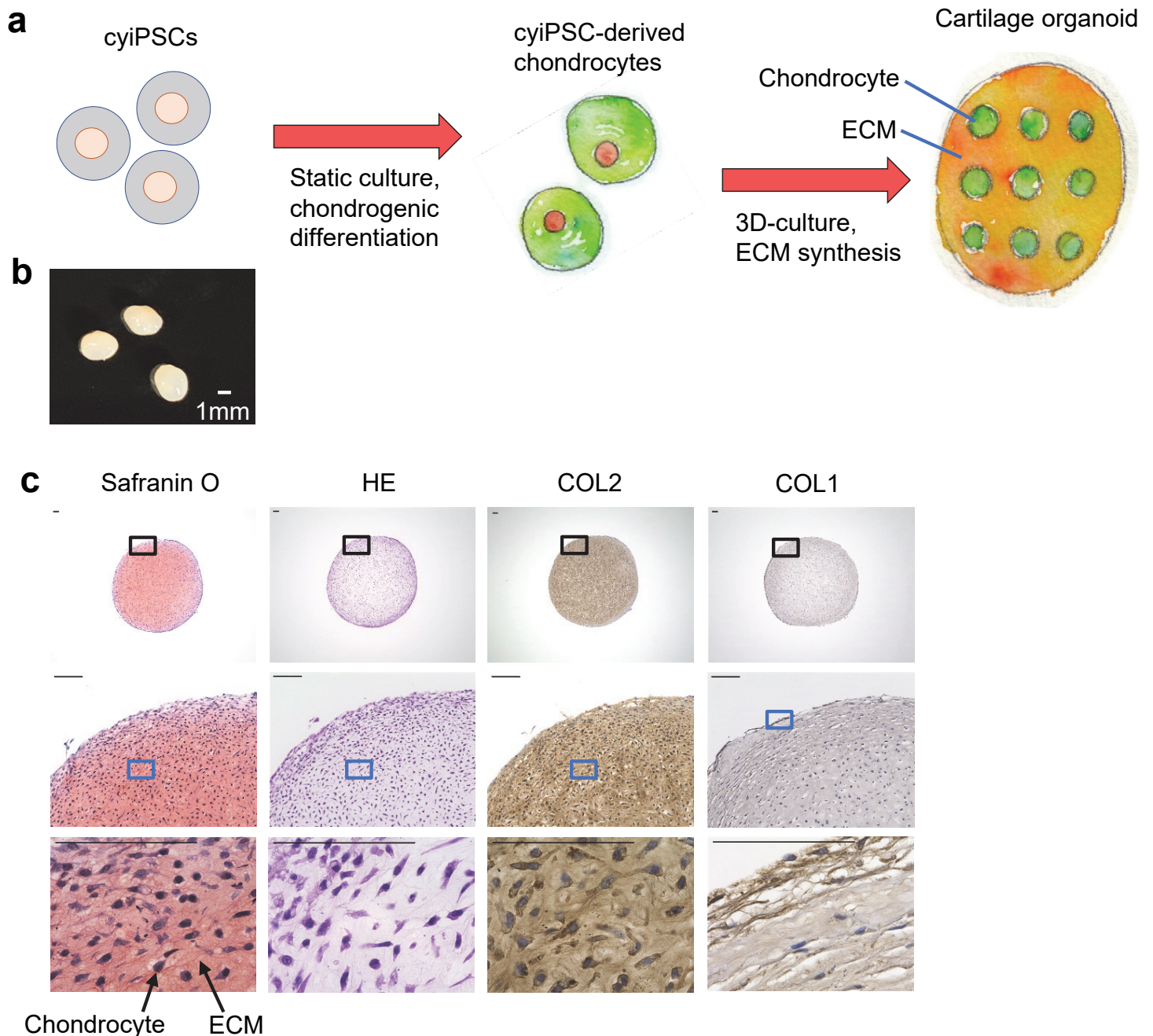
Figure 8



Supplementary Information

Engraftment of allogeneic iPS cell-derived cartilage organoid in a primate model of articular cartilage defect

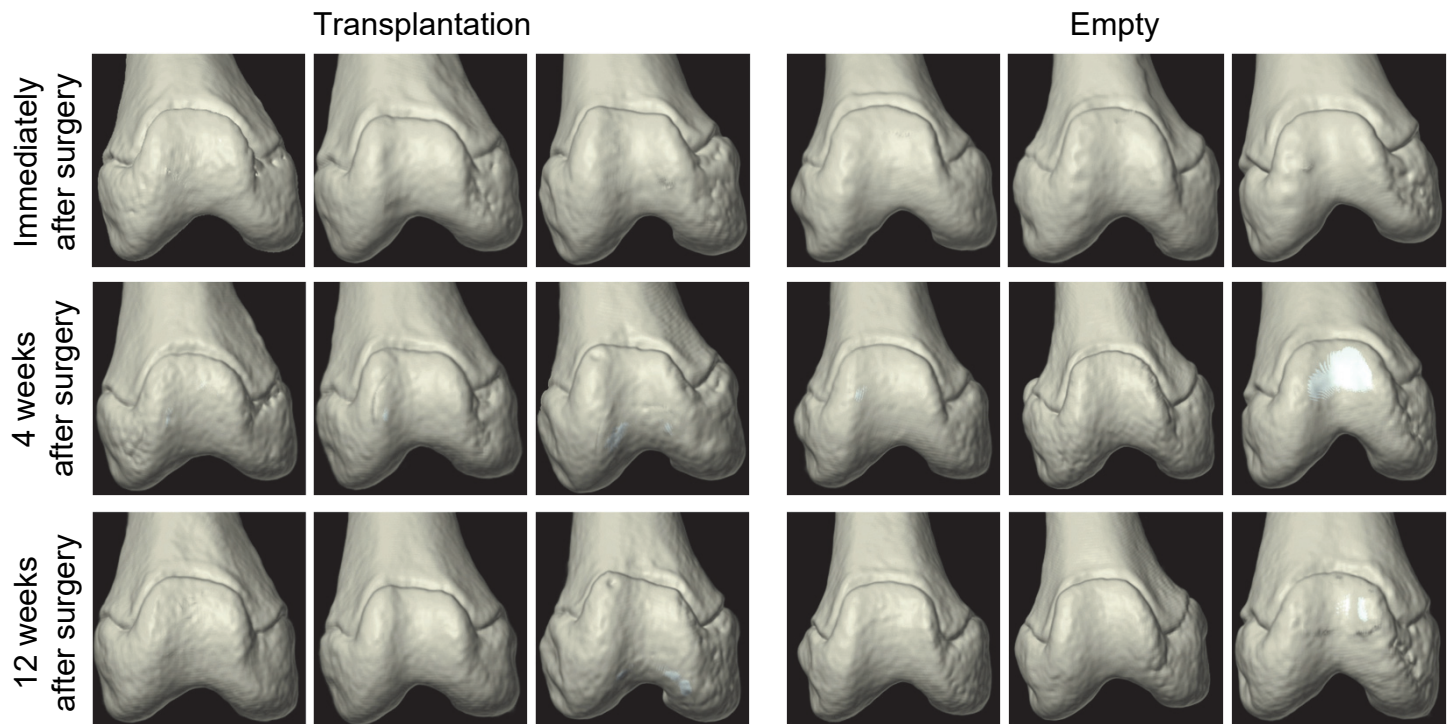
Kengo Abe, Akihiro Yamashita, Miho Morioka, Nanao Horike, Yoshiaki Takei, Saeko Koyamatsu, Keisuke Okita, Shuichi Matsuda, and Noriyuki Tsumaki



Supplementary Figure 1. cyiPS cell-derived cartilage organoids (cyiPS-Cart)

- Generation of cyiPS-Cart particle from cyiPSCs.
- Appearance of a cyiPS-Cart particle. Scale bar, 1 mm.
- Semi-serial histological sections of cyiPS-Cart particles were stained with safranin O-fast green-iron hematoxylin (safranin O) and hematoxylin-eosin (HE) and immunostained for type II collagen (COL2) and type I collagen (COL1). The black boxed regions in the top row are magnified in the middle row. The blue boxed regions in the middle row are magnified in the bottom row. Scale bars, 100 μ m.
Data are representative of three cyiPS-Cart organoids.

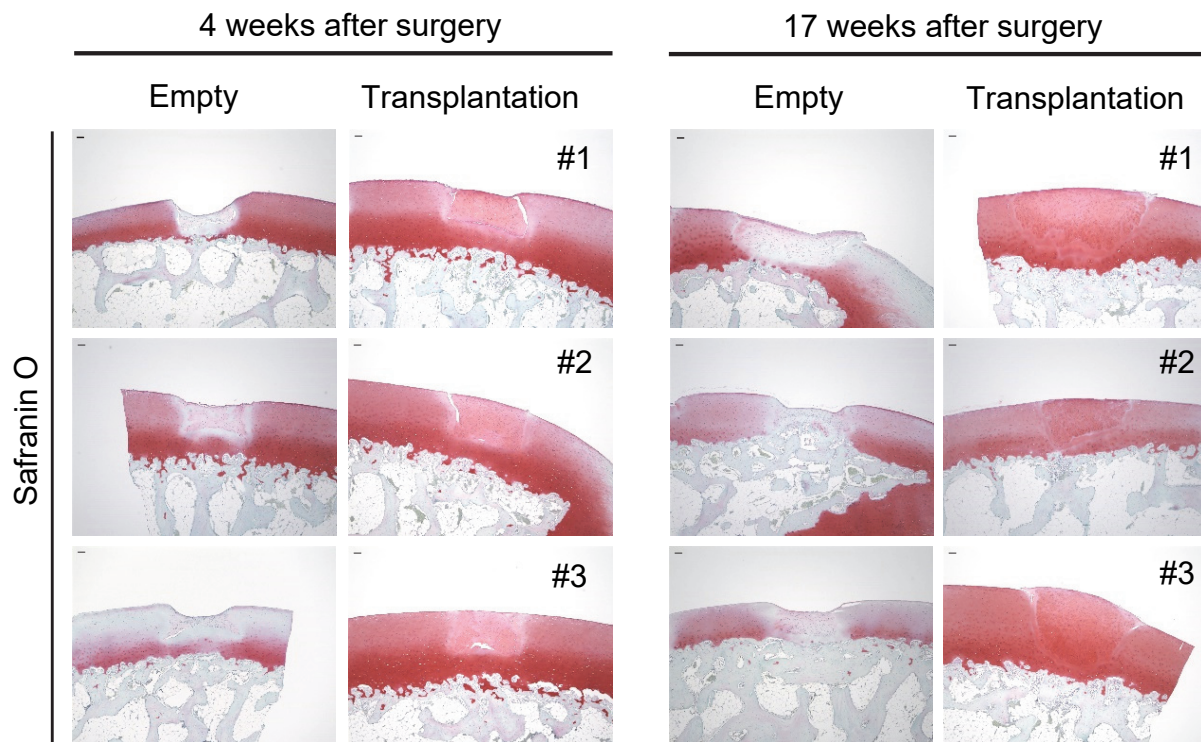
Supplementary Figure 2



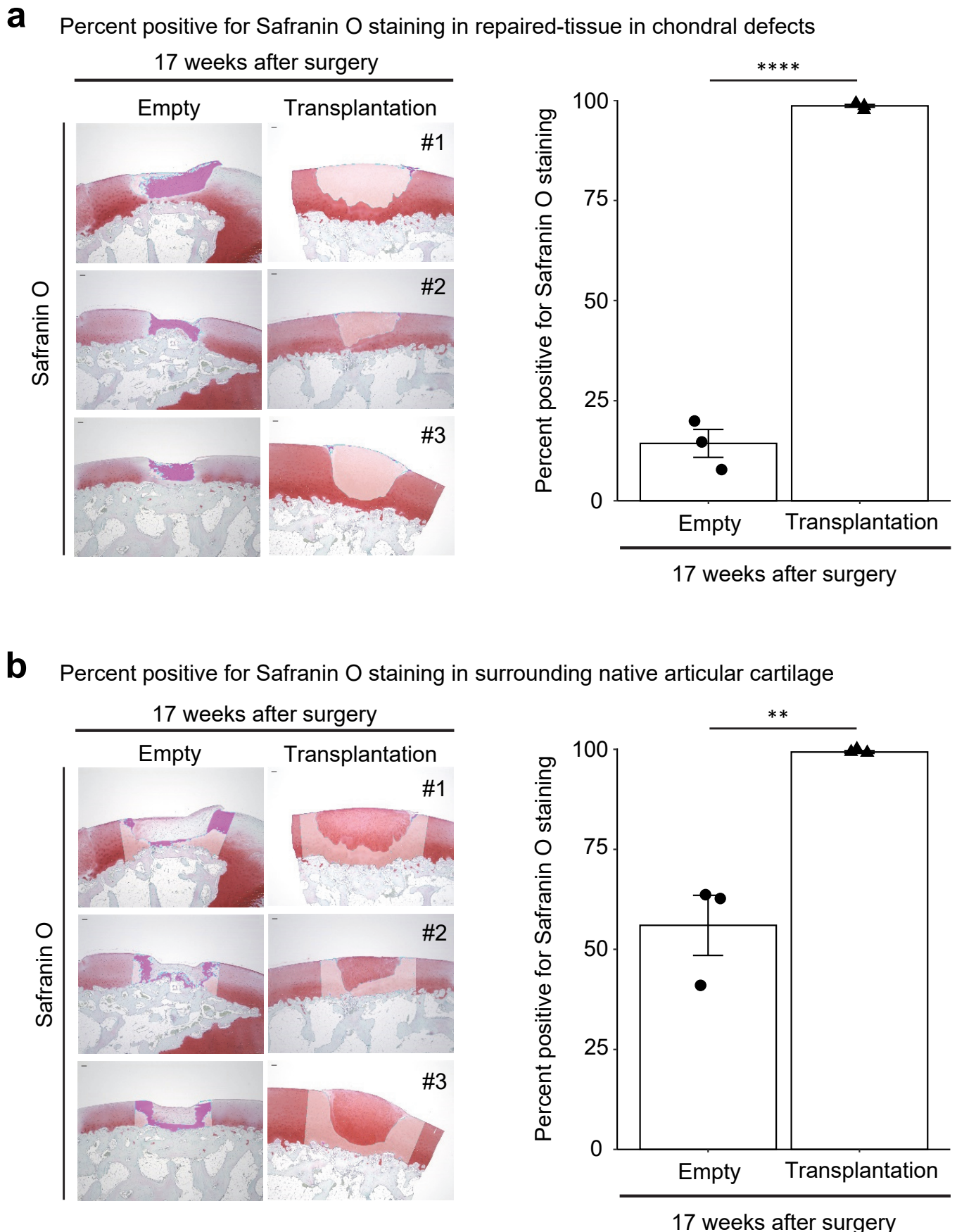
Supplementary Figure 2. Three-dimensional CT images of the knee joints after surgery.

CT was performed on all 12 monkeys immediately and 4 weeks after surgery. Six monkeys were sacrificed 4 weeks after surgery, and CT was performed on remaining monkeys 12 weeks after surgery. Images from the 6 monkeys that were sacrificed 17 weeks after surgery are shown.

Supplementary Figure 3



Supplementary Figure 3. Images of histological samples from all 12 monkeys. Each panel represents a different monkey. One representative image for each group is shown in Fig. 3a. Safranin O-fast green-iron hematoxylin staining. Scale bars, 100 μ m.



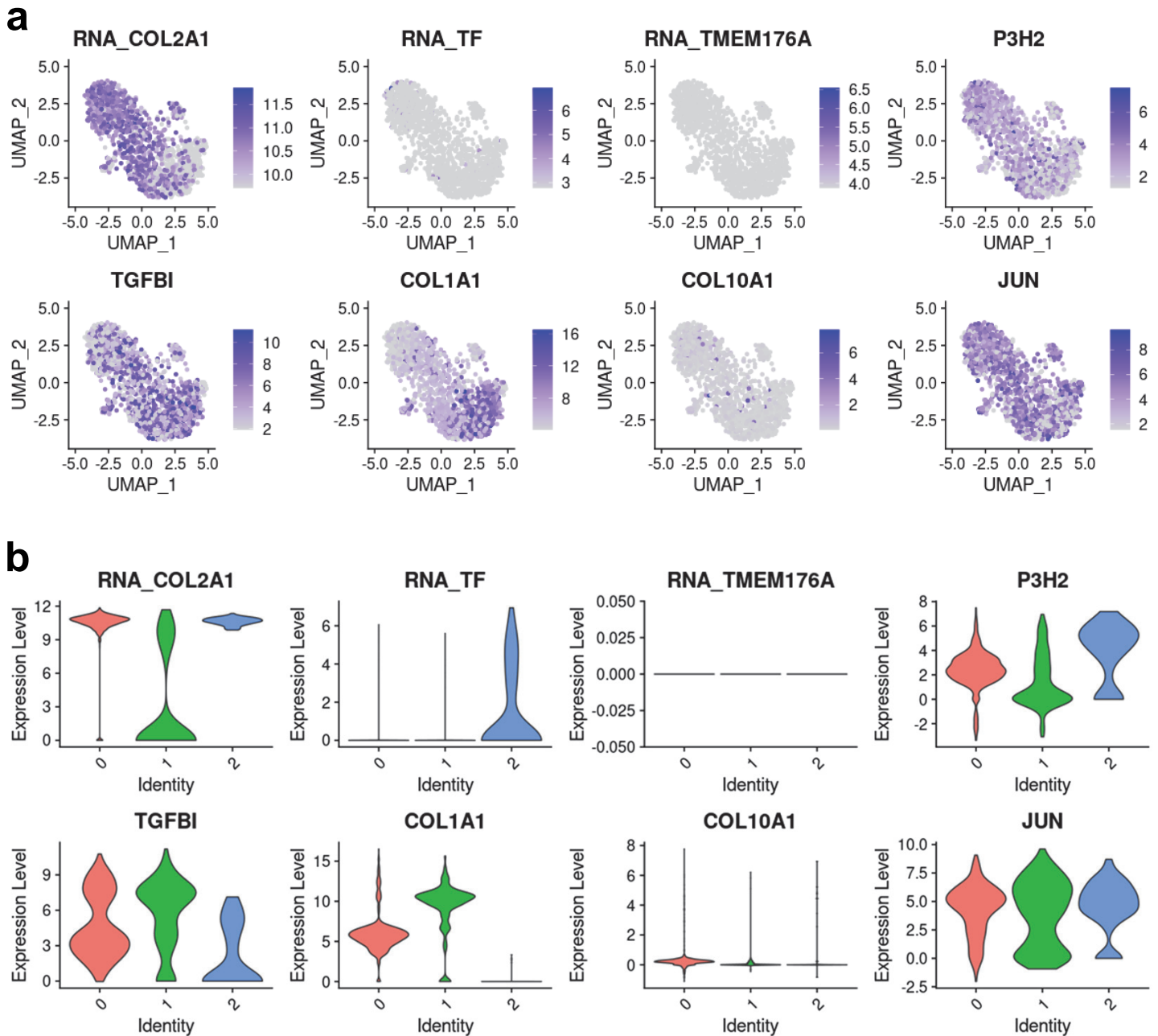
Supplementary Figure 4. Quantitative analysis of safranin O-positive area in repaired tissue in chondral defects and the surrounding native articular cartilage.

- a) The safranin O-positive area and total area of the repaired tissues in chondral defects were measured. The safranin O-positive area was divided by the total area.
- b) The safranin-O-positive area and the total area of native articular cartilage regions surrounding chondral defects were measured. The safranin O-positive area was divided by the total area.

Scale bars, 100 μ m. Error bars denote mean $\pm$ SE. **** P < 0.0001, ** P = 0.0045 by two-tailed Student's t -test (n = 3 monkeys).

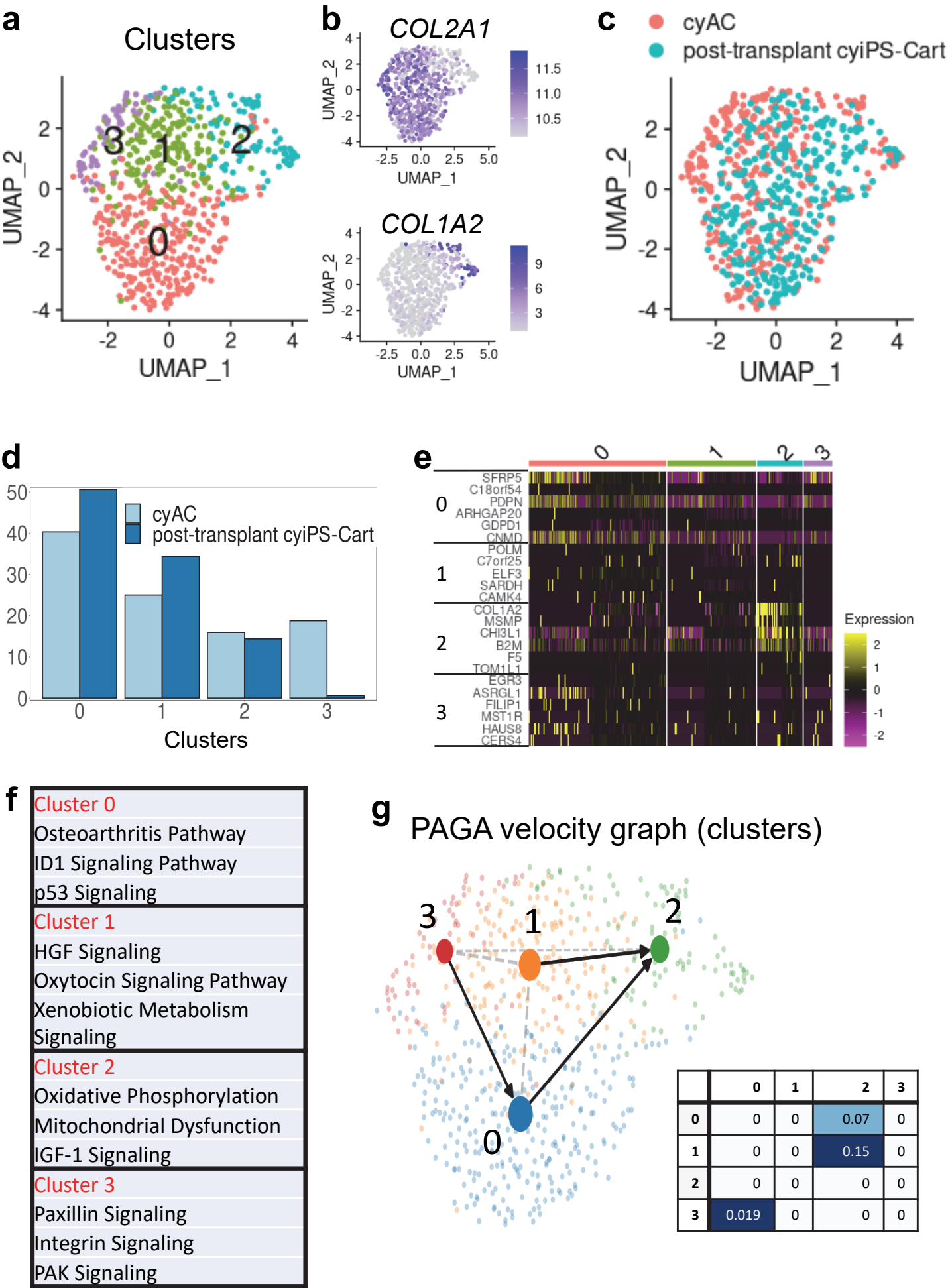
Source data are provided as a Source Data file.

Supplementary Figure 5



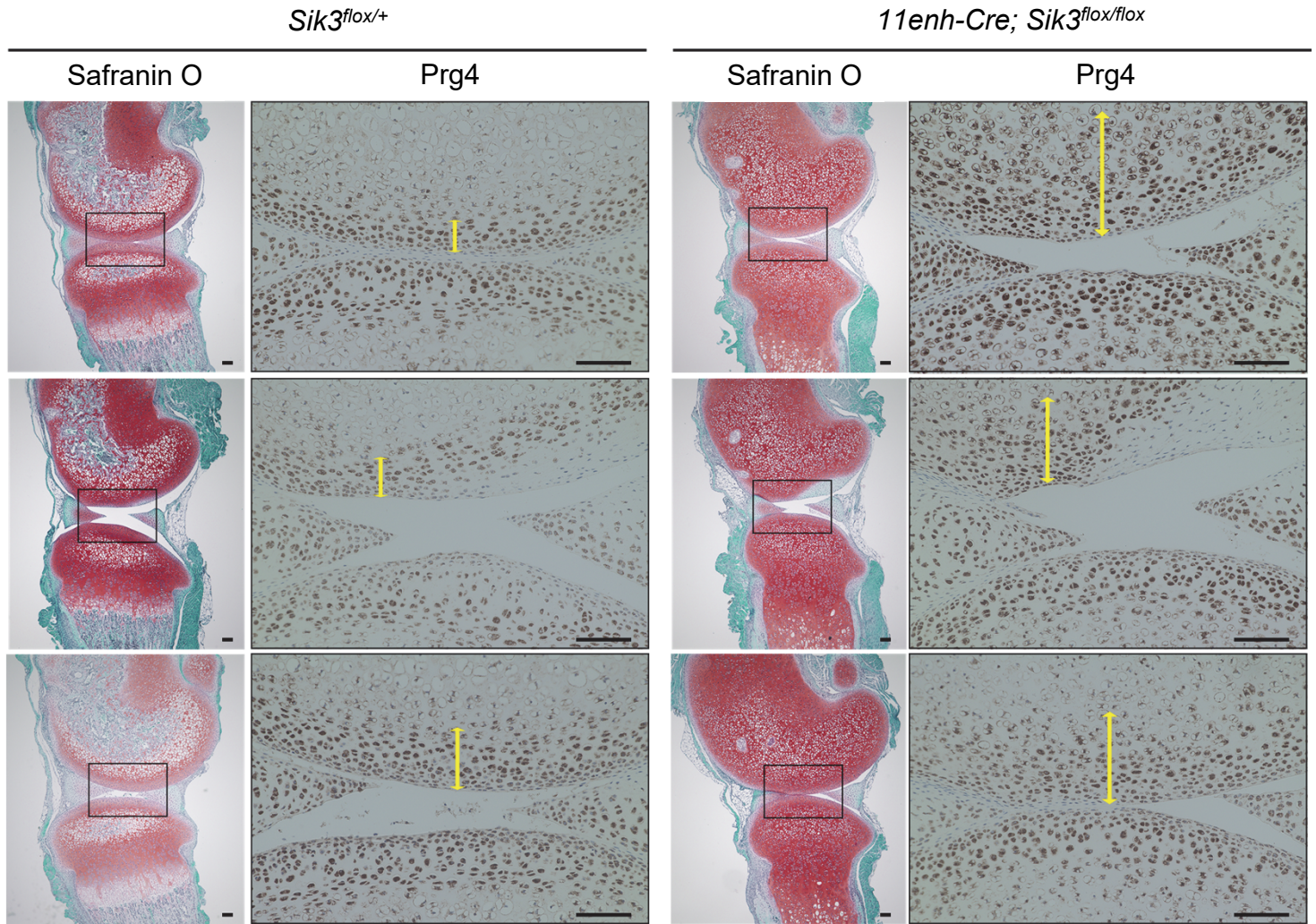
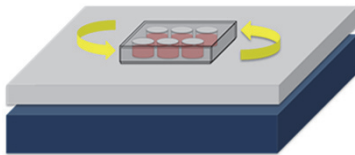
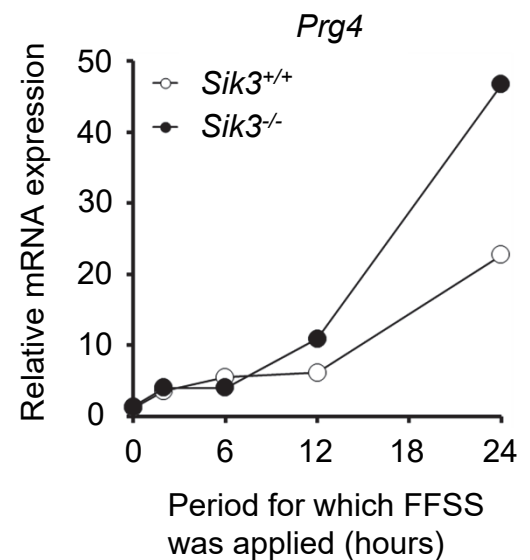
Supplementary Figure 5. scRNA-seq analysis of cyAC, cyFT, pre-transplant cyiPS-Cart, and post-transplant cyiPS-Cart.

- Expression levels of *COL2A1* and osteoarthritis marker genes are indicated in each cell projected on the UMAP plot using the feature plot function.
- Expression levels of *COL2A1* and osteoarthritis marker genes are indicated in each cell projected on the UMAP plot using the “VlnPlot” function. The results of Clusters #3 and #4 were omitted because cell numbers in these clusters are few.



Supplementary Figure 6. scRNA-seq analysis of cyAC, cyF and post-transplant cyiPS-Cart.

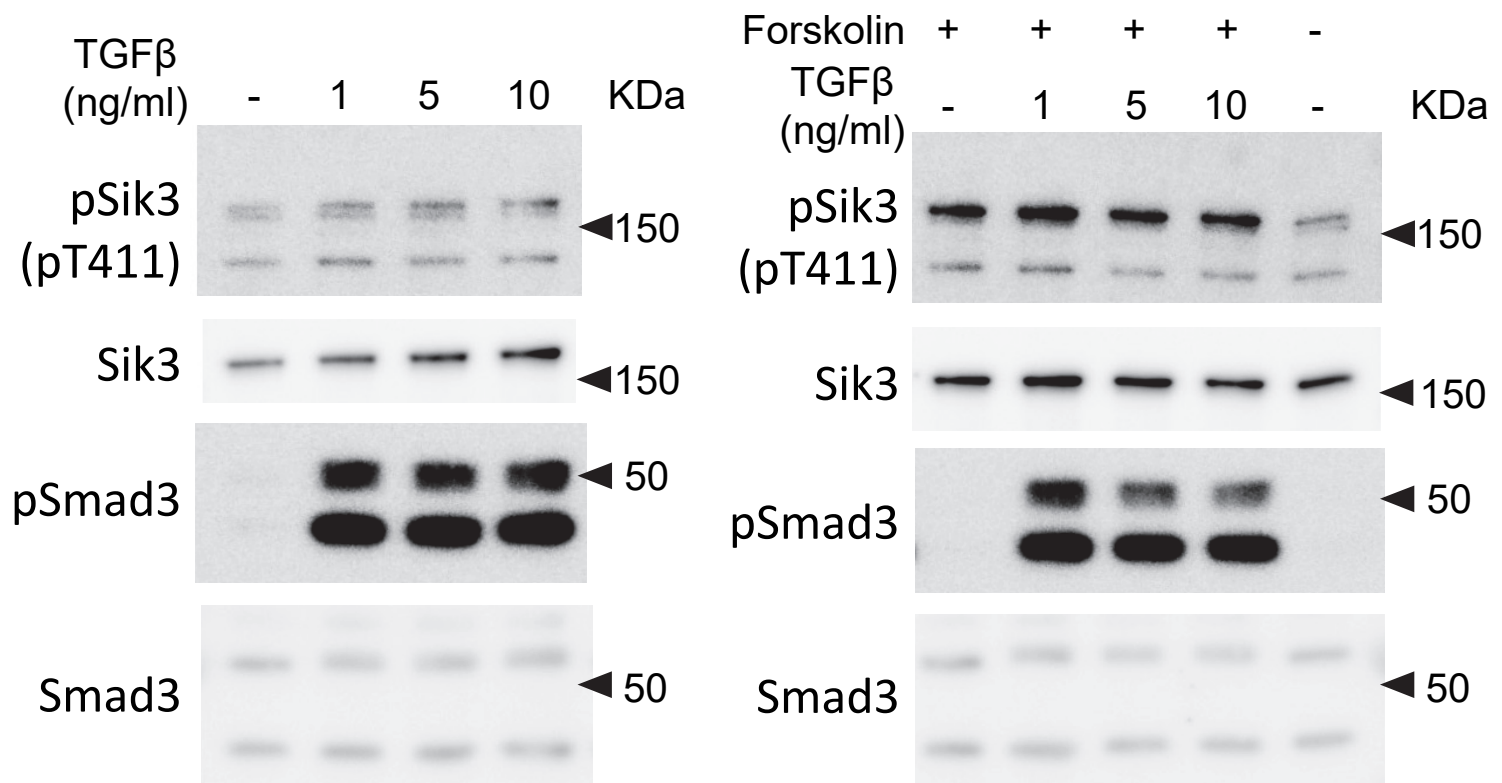
- a) After reducing the cell number for each sample to 320, the data from the samples were integrated. The cells were then clustered with a parameter resolution of 0.95 and projected onto the UMAP plots.
- b) *COL2A1* and *COL1A2* expression levels are indicated in each cell projected on the UMAP plot using the feature plot function.
- c) Distribution of cells in each sample is indicated on the UMAP plot.
- d) The ratio of the number of cells in each cell cluster in each sample in (a) is plotted.
- e) Heatmap revealing the scaled expression of differentially expressed genes for each cluster defined in (a).
- f) Canonical pathways enriched for each cluster based on differentially expressed genes.
- g) Post-transplant cyiPS-Cart cells were subjected to RNA velocity analysis using scVelo. The trajectory inference using PAGA was extended by velocity-inferred directionality.

a**b****c**

Supplementary Figure 7. Analysis of shear stress and *Sik3* contribution to *Prg4* expression.

- Images of histological samples from other three conditional knockout mice and three *Sik3^{lox/+}* mice for Fig. 8d. Scale bars: 100 μ m.
- Schematic representation of the orbital shaker used to subject primary chondrocytes to FFSS.
- The other data of two independent experiments for Fig. 8e. Source data are provided as a Source Data file.

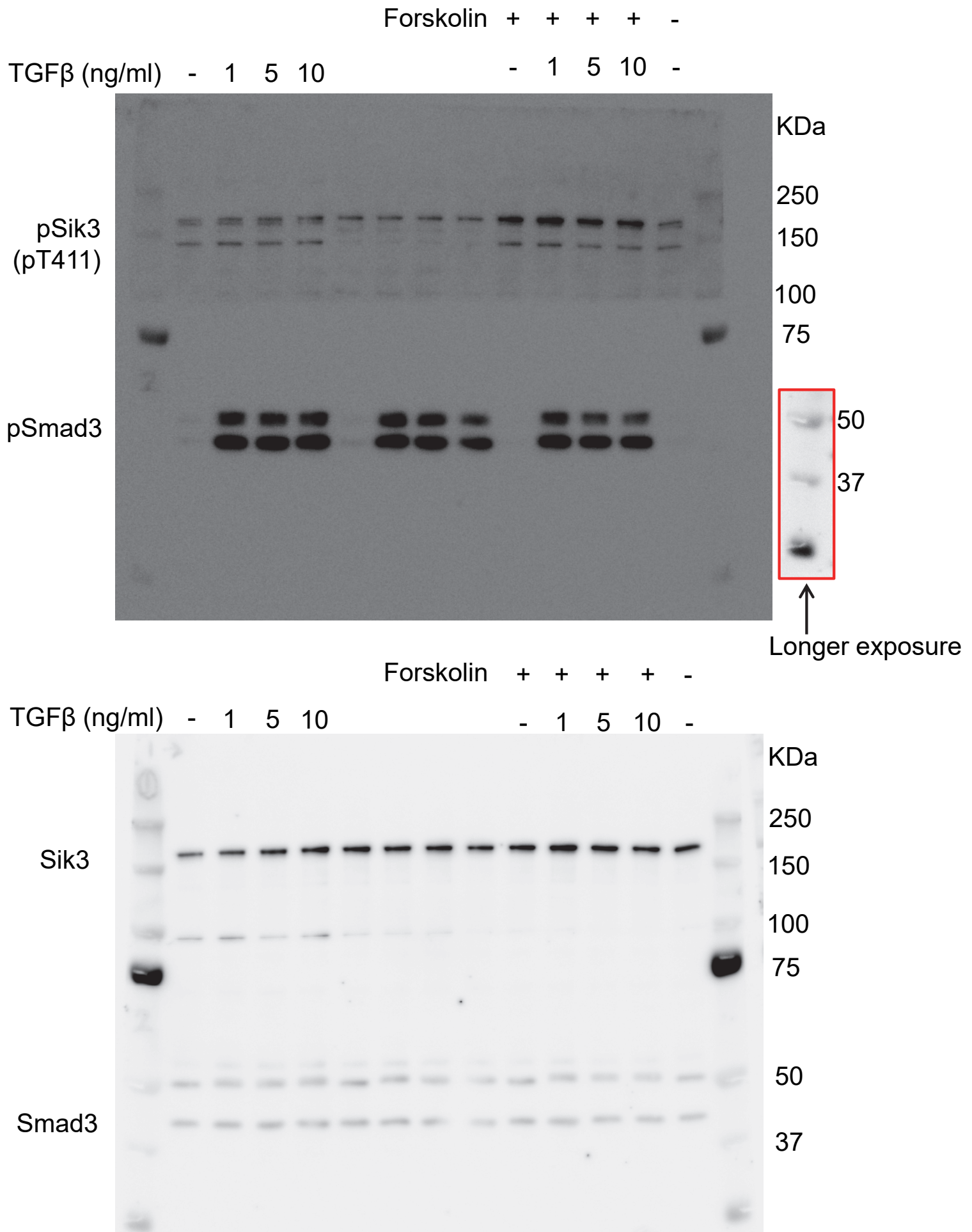
Supplementary Figure 8



Supplementary Figure 8. Relationship between TGF-β signaling and Sik3 in chondrocytes.

Mouse primary chondrocytes were treated with or without 1, 5 or 10 ng/mL TGF-β1 (*left*), or treated with or without 1, 5 or 10 ng/mL TGF-β1 or 100 μg/mL forskolin (*right*) for 1 h for immunoblot analysis. Data are representative of two independent experiments. Uncropped images are provided in Supplementary Figure 9.

Supplementary Figure 9



Supplementary Figure 9 Uncropped immunoblot images for Supplementary Fig. 8.

Supplementary Table 1. MHC genotypes

	cyiPSC line (Donor)*	Sacrificed at 4 weeks after surgery			Sacrificed at 17 weeks after surgery		
		#1	#2	#3	#1	#2	#3
Mafa-F	F-like4	F-like2	F-like2		F-like2	F-like2 F-like4	F-like7
Mafa-A	A1*052:02 A4*01:04	A1*040:03 A1*053:01/02 A2*05:21 A3*13:16 A4*01:07 A4*03:02	A1*040:01 A1*065:03 A3*13:07 A3*13:16 A4*03:02		A1*022:05 A1*040:03 A2*05:38 A4*14:15	A1*073:01 A1*096:01 A2*05:59 A4*14:01/08/10 A4*14:03/04/13	A1*018:01/09 A1*060:04 A1*086:01 A3*13:02
Mafa-E	E-like5 E-like11	E-like3 E-like6 E-like14	E-like1 E-like3		E-like1 E-like6	E-like3 E-like7/10 E-like11 E-like14	E-like1 E-like4
Mafa-B	B*095:01 B*033:02 B*098:10	B*021:01/05/07 B*028:03/05/06 B*028:04 B*051:12 B*054:02/03 B*060:04/13/19 B*060:23 B*068:02 B*082:05 B*105:01 B*124:05N B*149:02 B*156:01 B*205:01	B*050:01/02/06/08/10/11/12 B*051:05/08 B*060:04/13/19 B*065:02/04 B*069:05 B*072:07 B*088:05 B*117:03 B11L*01:01	n.d.**	B*015:03/08 B*036:01/04 B*037:01 B*045:07 B*050:01/02/06/08/10/11/12 B*051:04/10/16 B*068:06/07/11/13 B*079:04 B*109:03 B*115:05 B*167:01N B*180:01/03 B*202:01	B*036:01:01/02/04 B*037:01 B*045:07 B*046:12 B*050:01/02/06/08/10/11/12 B*098:08 B*105:01 B*149:02 B*156:01 B*167:01N B*180:01/03	B*007:01/07/09/11 B*014:01/02 B*034:03 B*050:05 B*051:05/08 B*060:04/13/19 B*097:01 B*174:01N
Mafa-I	I*01:11	I*01:27	I*01		I*01	I*01:27	I*01
Mafa-DRB	DRB1*10:07 DRB1*03:21	DRB1*03:12/36 DRB*W002:05 DRB*W020:03 DRB*W025:01	DRB1*03:12/36 DRB*W021:03 DRB*W026:01 DRB*W027:03		DRB1*03:03/30 DRB1*03:12/36 DRB*W001:01 DRB*W002:03 DRB*W003:02	DRB1*03:03/30 DRB1*03:12/36 DRB*W001:02 DRB*W002:06 DRB*W026:01	DRB1*03:12/36 DRB*W002:03 DRB*W021:01 DRB*W027:01:01
Mafa-DQA1	DQA1*01:07:01	not done	not done		not done	not done	not done
Mafa-DQB1	DQB1*06:08	DQB1*15:03 DQB1*18:09	DQB1*06:14 DQB1*18:07/26		DQB1*15:01 DQB1*17:03/12	DQB1*06:14 DQB1*17:03	DQB1*15:01 DQB1*17:02
Mafa-DPA1	DPA1*02:05	not done	not done		not done	not done	not done
Mafa-DPB1	DPB1*15:04	DPB1*05:01 DPB1*19:01	DPB1*15:01/13 DPB1*19:01		DPB1*03:03/04/05/06 DPB1*05:01	DPB1*01:04/08/10/15 DPB1*19:06:01	DPB1*03:03/04/05 DPB1*05:01

*The cyiPSC line had homozygous MHC haplotype.

**Not determined because the blood sample of this monkey was mistaken.

Supplementary Table 2. Modified Wakitani histological scoring system

Category points	Scores
Cell morphology	
Hyaline cartilage	0
Mostly hyaline cartilage	1
Mostly fibrocartilage	2
Mostly noncartilage	3
noncartilage	4
Matrix staining with safraninO and fast green	
Normal (compared with host adjacent cartilage)	0
Slightly reduced	1
Markedly reduced	2
No metachromatic stain	3
Thickness of cartilage	
>2/3	0
1/3-2/3	1
<1/3	2
Integration of implant with adjacent host cartilage	
Both edges integrated	0
One edge integrated	1
Neither edge integrated	2
Total maximum	11

Supplementary Table 3. List of antibodies used for immunohistochemistry

Antibody	Species	Dilution	Catalog No.	Source
Anti Collagen I	Goat	1:1500	1310-01	Southern Biotech
Anti Collagen II	Goat	1:300	1320-01	Southern Biotech
Anti Lubricin/Proteoglycan 4	Mouse	1:500	MABT400	Milipore
Anti CD3	Rat	1:100	ab11089	abcam
Anti GFP	Rabbit	1:500	NB600-308	NOVUS
Anti mouse immunoglobulin HRP		No dilution	K1497	DAKO
Anti rabbit immunoglobulin HRP	Mouse	1:100	sc-2357	Santa Cruz Biotechnology
Anti goat immunoglobulin HRP	Donkey	1:1000	ab97110	abcam
Alexa Fluor 488 goat anti rat IgG(H+L)	Goat	1:1000	A11006	Invitrogen

Supplementary Table 4. List of primers

Target		F/R	Sequence 5'→3'
monkey	GAPDH	F	CCTTCACACCCTTGCGTATT
monkey	GAPDH	R	TTGATTTTGGAGGGATCTCG
monkey	PRG4	F	GGAGATGTGGGGAAGGGTAT
monkey	PRG4	R	TGCTTTCTTTGCAGATGGTG
mouse	GAPDH	F	TGGATTTGGACGCATTGGTC
mouse	GAPDH	R	TTTGCACTGGTACGTGTTGAT
mouse	ACTB	F	GGCTGTATTCCCCTCCATCG
mouse	ACTB	R	CCAGTTGGTAACAATGCCATGT
mouse	PRG4	F	TGGAGTGCTGTCCTGATTTCAAGAG
mouse	PRG4	R	GGTGATTTGGGTGAGCGTTTGGTA