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1 **The differentiation of prehypertrophic into hypertrophic chondrocytes**
2 **drives an OA-remodeling program and IL-34 expression**

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26

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Abstract

29

30

31 **Objectives.** We hypothesize that chondrocytes from the deepest articular cartilage layer
32 are pivotal in maintaining cartilage integrity and that the modification of their
33 prehypertrophic phenotype to a hypertrophic phenotype will drive cartilage degradation in
34 osteoarthritis.

35 **Design.** Murine immature articular chondrocytes (iMACs) were successively cultured into
36 three different culture media to induce a progressive hypertrophic differentiation.
37 Chondrocyte were phenotypically characterized by whole-genome microarray analysis.
38 The expression of IL-34 and its receptors PTPRZ1 and CSF1R in chondrocytes and in
39 human osteoarthritis tissues was assessed by RT-qPCR, ELISA and
40 immunohistochemistry. The expression of bone remodeling and angiogenesis factors and
41 the cell response to IL-1 β and IL-34 were investigated by RT-qPCR and ELISA.

42 **Results.** Whole-genome microarray analysis showed that iMACs, prehypertrophic and
43 hypertrophic chondrocytes each displayed a specific phenotype. IL-1 β induced a stronger
44 catabolic effect in prehypertrophic chondrocytes than in iMACs. Hypertrophic
45 differentiation of prehypertrophic chondrocytes increased Bmp-2 (95%CI [0.78;1.98]),
46 Bmp-4 (95%CI [0.89;1.59]), Cxcl12 (95%CI [2.19;5.41]), CCL2 (95%CI [3.59;11.86]),
47 Mmp3 (95%CI [10.29;32.14]) and Vegf mRNA expression (95%CI [0.20;1.74]).
48 Microarray analysis identified IL-34, PTPRZ1 and CSFR1 as being strongly overexpressed
49 in hypertrophic chondrocytes. IL-34 was released by human osteoarthritis cartilage; its
50 receptors were expressed in human osteoarthritis tissues. IL-34 stimulated CCL2 and
51 MMP13 in osteoblasts and hypertrophic chondrocytes but not in iMACs or
52 prehypertrophic chondrocytes.

53 **Conclusion.** Our results identify prehypertrophic chondrocytes as being potentially pivotal
54 in the control of cartilage and subchondral bone integrity. Their differentiation into

55 hypertrophic chondrocytes initiates a remodeling program in which IL-34 may be
56 involved.

57

58 **Keywords:** Osteoarthritis, prehypertrophic chondrocytes, osteochondral junction,
59 chondrocyte hypertrophic differentiation, IL-34

Introduction

60

61

62 Osteoarthritis (OA) is characterized by the irreversible degradation of cartilage, which is
63 associated with a pathological remodeling of the subchondral bone, including sclerosis and
64 osteophyte formation. Cartilage degradation mainly results from the proteolysis of the
65 cartilage extracellular matrix by chondrocyte-secreted proteases ¹. The degradation
66 observed in the deep zone of articular cartilage is explained by an endochondral
67 ossification-like process at the cartilage/bone interface ²⁻⁴, which involves the hypertrophic
68 differentiation of chondrocytes, the calcification and the vascularization of the extracellular
69 matrix followed by the replacement of cartilage with bone.

70 While chondrocytes are the unique cell type present within cartilage, different
71 chondrocyte phenotypes exist, depending on the type of cartilage and on the chondrocyte
72 localization within cartilage. Articular cartilage is organized in different layers from the
73 surface until the subchondral bone. The phenotype of chondrocytes differs upon the layer
74 considered ⁵. Chondrocytes from the deepest articular cartilage layer of non-calcified
75 cartilage display an intermediate phenotype between that of the chondrocytes of the surface
76 layers and that of the chondrocytes found in the calcified cartilage, which are hypertrophic.
77 They indeed express molecules that characterize both surface layer chondrocytes and
78 hypertrophic chondrocytes, including type II and type X collagens ⁶⁻⁸. They also express
79 Ihh and osteomodulin as prehypertrophic chondrocytes from the growth plate cartilage ⁹⁻¹².

80 Cartilage degradation in OA results in chondrocyte phenotypic modifications. We
81 hypothesize that the chondrocytes from the deepest articular cartilage layer play a crucial
82 role in maintaining cartilage integrity and that the modification of their prehypertrophic
83 phenotype to a hypertrophic phenotype will drive cartilage degradation in OA. In the
84 present study, we developed a model of progressive differentiation of murine immature

85 articular chondrocytes (iMACs) into hypertrophic chondrocytes, and this model includes
86 an intermediate prehypertrophic state. Here, we show that the differentiation of
87 prehypertrophic chondrocytes into hypertrophic chondrocytes shifts chondrocytes towards
88 an OA-inducing phenotype. This phenotype is associated with an increased expression of
89 IL-34, a recently discovered cytokine that could be involved in both cartilage and bone
90 integrity.

91

Materials and methods

92

93

94 See supplementary information for detailed Material and methods.

95

96 **Collection of osteoarthritis human samples**

97 Human OA knee explants (n=33) obtained from patients undergoing total knee joint
98 replacement surgery were dissected, as described ¹³.

99

100 **Immunohistochemistry**

101 Immunohistochemistry was performed with a mouse monoclonal antibody against
102 PTPRZ1 (clone 12/RPTPb, BD Transduction Laboratories; dilution 1:50) and a rabbit
103 polyclonal antibody against CSF-1R (H-300, Santa Cruz Biotechnology; dilution 1:50) as
104 the primary antibodies. The R.T.U. Vectastain kit (Vector) was used for detection,
105 followed by counterstaining with Mayer's hematoxylin. Irrelevant control antibodies
106 (Dako) were incubated at the same concentration to assess nonspecific staining.

107

108 **Primary culture of murine osteoblasts and articular chondrocytes**

109 Osteoblasts and iMACs were isolated and cultured, as described in ¹⁴⁻¹⁶ and
110 supplementary information. Prehypertrophic chondrocytes were obtained by culturing
111 iMACs for 28 days in culture medium 2 (DMEM/HAM-F12 medium supplemented with
112 fetal calf serum (5%), penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (4
113 mM), ascorbic acid (40 µg/mL), insulin-transferrin-sodium selenite (1%) and triiodo-L-
114 thyronine (50 ng/mL)). Chondrocytes were further cultured for 42 days in medium 2
115 supplemented with β-glycerophosphate (10 mM), retinoic acid (100 nM) and 1α,25-
116 dihydroxyvitamin D₃ (10 nM) (Medium 3) to obtain hypertrophic chondrocytes. All

117 cultures were performed in standard conditions with the exception of the last
118 differentiation step, performed in 3% CO₂/95% air. At the end of the culture, cells were
119 serum-starved for 24 hours and stimulated by recombinant human IL-1 β (1 ng/mL) or
120 murine IL-34 (3, 30 and 100 ng/mL) for 24 hours. Conditioned media were kept,
121 centrifuged and stored at -20°C. Cells were either fixed in 3.7% paraformaldehyde (PFA)
122 or used for mRNA or protein extraction.

123 The experimental design for the cell culture study is shown in Fig. S1.

124

125 **Primary culture of human articular chondrocytes**

126 Human chondrocytes were isolated from the less damaged areas of OA cartilage from
127 patients who underwent total knee arthroplasty. Their hypertrophic differentiation was
128 performed according to Yahara *et al.* ¹⁷.

129

130 **Microarray analysis**

131 mRNA expression profiling was performed using SurePrint G3 Mouse Gene Expression
132 v2 8x60K Microarray (G4852B, Agilent Technologies) and SurePrint Mouse miRNA
133 Microarray Kit v21 8x60K (G4859C, Agilent Technologies). For mRNA profiling, probe
134 labeling and 60 mer-oligonucleotide microarray hybridization were performed according to
135 the manufacturer's instructions ¹⁸. An Agilent scanner and Feature Extraction 11.5.1.1
136 software (Agilent Technologies) were used to obtain the raw microarray data for both
137 analyses.

138

139

140 **Statistical analysis**

141 We used repeated measures one-way ANOVA to compare iMACs, prehypertrophic and
142 hypertrophic chondrocytes. Paired t-tests were used to compare prehypertrophic to
143 hypertrophic chondrocyte gene expression observations and gene or protein comparison in
144 OA patients. For the IL-1 β and IL-34 stimulation study, we used Dunnett's post hoc test.
145 The analyses were performed using GraphPad Prism7 (GraphPad Software Inc., San Diego,
146 CA, USA).

147

Results

148

149

150 **Model of the progressive differentiation of murine immature articular chondrocytes** 151 **into prehypertrophic and hypertrophic chondrocytes**

152 To study the consequences of the hypertrophic differentiation of chondrocytes in OA
153 cartilage, we established an *in vitro* model of the progressive differentiation of iMACs into
154 prehypertrophic and hypertrophic chondrocytes. iMACs displayed the typical phenotype of
155 articular chondrocytes, characterized by the expression of Col2a1, Acan, Chm1 and Sox9
156 and by the almost complete absence of the expression of the hypertrophic chondrocyte
157 markers Runx2, Osterix, MMP-13, Col10a1 and Tnap (n=6) (Fig. 1B-E and I-M). iMACs
158 also showed weak alkaline phosphatase activity and no evidence of matrix calcification
159 (Fig. 1O and P). Culturing the iMACs in Medium 2 then in Medium 3 induced progressive
160 decreases in the mRNA expression of Acan, Col2a1, Sox9 and Chm1 (Fig. 1B-E).
161 Inversely, the mRNA expression of the hypertrophic markers Runx2, Osterix, Mmp13,
162 osteocalcin and Tnap was stimulated by culture in Medium 2 (Fig. 1I-N). The mRNA
163 expression of Runx2, Col10a1 and osteocalcin further increased after the culture in
164 Medium 3. Consistent with the increased mRNA expression of Tnap, the activity of
165 alkaline phosphatase was strongly stimulated by culture in Medium 2 and Medium 3 (Fig.
166 1O). Significant calcification of the chondrocyte cultures was only observed when cells
167 were cultured in Medium 3 (Fig. 1P). Cells cultured in Medium 2 expressed both iMAC
168 and hypertrophic chondrocyte markers and did not mineralize their matrix. They also
169 expressed the markers of prehypertrophic chondrocytes Ihh, Snorc and Osteomodulin (Fig.
170 1F-H). Moreover, they expressed higher mRNA levels of Snorc and Osteomodulin than
171 iMACs and hypertrophic chondrocytes. Thus, iMACs progressively became
172 prehypertrophic and hypertrophic after culture in Medium 2 and Medium3.

173 To further characterize our model of progressive hypertrophic differentiation of iMACs,
174 the transcriptomic signatures of iMACs, prehypertrophic and hypertrophic chondrocytes
175 were explored by high-throughput genomic methods (n=8). A two-dimensional PCA of the
176 genes expressed revealed that all displayed globally distinct gene expression patterns (Fig.
177 S2B), which were also confirmed by their distinct clustering patterns (Fig. S2C).
178 Interestingly, prehypertrophic and hypertrophic chondrocytes showed a homogenous
179 clustering pattern of gene expression, indicating that they displayed a specific molecular
180 phenotype, different from iMAC phenotype.

181 The most variable genes identified by PCA (top 5000 ranked by decreasing standard
182 deviation) accounted for 40% and 17% of the total gene expression variability in the
183 principal component (PC) 1 and PC2 groups, respectively. Genes positively correlated with
184 PC1 displayed an upregulation across the differentiation process. The number of genes
185 positively correlated with PC2 was more restricted, and those were of particular interest to
186 characterize prehypertrophic cells since they were more specifically associated with this
187 group of cells.

188 The repeated spotted probes and the probes targeting the same gene were not averaged
189 but were analyzed for similar expression. We identified 8121 differentially expressed (DE)
190 genes (9306 DE probes, at false discovery rate (FDR)-adjusted p-value ≤ 0.05 & $|\text{Fold}$
191 $\text{Change (FC)}| \geq 1.3$) between iMACs and hypertrophic chondrocytes (4311 and 3810 genes
192 overexpressed in hypertrophic chondrocytes and iMACs, respectively), including markers
193 of articular and hypertrophic chondrocytes (Table S1). The specific part of this signature
194 represented 1467 genes (1768 DE probes, 809 and 959 overexpressed in hypertrophic
195 chondrocytes and iMACs, respectively) (Fig. 2A). We found 6829 differentially expressed
196 genes (7744 probes) between prehypertrophic chondrocytes and iMACs (3601 and 3228
197 overexpressed in prehypertrophic chondrocytes and iMACs, respectively) (Table S1),

198 while 1023 DE probes were specific to this contrast (540 and 483 overexpressed in
199 prehypertrophic chondrocytes and iMACs, respectively) (Fig. 2B). Finally, 5308 genes
200 (6220 probes) were differentially expressed between the hypertrophic and prehypertrophic
201 chondrocytes (2970 and 2338 genes overexpressed in hypertrophic and prehypertrophic
202 chondrocytes, respectively) (Table S1). Eight hundred nine genes (1002 DE probes) were
203 specific to differences between hypertrophic and prehypertrophic chondrocytes (626 and
204 376 probes overexpressed in hypertrophic and prehypertrophic chondrocytes, respectively)
205 (Fig. 2C). Hypertrophic chondrocytes were thus molecularly the most different from other
206 cells.

207 Together, these results show that our culture model allows a progressive differentiation
208 of the iMACs into prehypertrophic and hypertrophic chondrocytes, each displaying a
209 specific molecular phenotype.

210

211 **Prehypertrophic to hypertrophic differentiation shifts chondrocytes towards an OA-** 212 **inducing phenotype**

213 Inflammatory factors, such as IL-1 β , alter the phenotype of chondrocytes that adopt
214 OA-like catabolic features. As expected, IL-1 β downregulated the expression of Col2a1 by
215 iMACs (P_{adj}=0.0454, 95%CI [-1.41;-0.00], n=6), whereas it strongly upregulated the
216 mRNA expression of Il-6 (P_{adj}=0.0016, 95%CI [1.01;2.60], n=6) and Mmp13
217 (P_{adj}=0.0483, 95%CI [0.62;2.14], n=6) (Fig. 3A, C and D). IL-1 β also induced a similar
218 catabolic phenotype in prehypertrophic and hypertrophic chondrocytes (Fig. 3A-D).
219 However, prehypertrophic chondrocytes appeared more sensitive to IL-1 β than did iMACs
220 since IL-1 β led to a 2.0-fold, 4.1-fold and 1.8-fold lower expression of Col2a1, Acan and
221 Chm1 (n=6), respectively and to a 131.7-fold higher expression of Il-6, as compared to

222 iMACs (Fig. 3A-C). In addition, IL-1 β also stimulated the expression of Vegf and
223 repressed those of Tsp1 and Chm1 by prehypertrophic and hypertrophic chondrocytes
224 (n=6), while neither Vegf nor Tsp1 expression was regulated by IL-1 β in iMACs (Fig. 3E-
225 G).

226 To evaluate whether the shift from prehypertrophic to hypertrophic chondrocytes
227 mimicked OA-related osteochondral remodeling, we performed analysis focused on
228 molecular functions involved in OA (Fig. S2D). Hypertrophic chondrocytes, in contrast to
229 prehypertrophic chondrocytes, displayed activated functions related to bone differentiation,
230 angiogenesis and deterioration/damage of connective tissues. Accordingly, the mRNA
231 expression of the bone remodeling factors BMP-2 (2.38-fold, 95%CI [0.78;1.98] for the
232 difference in means), BMP-4 (2.25-fold, 95%CI [0.89;1.59] for the difference in means),
233 CXCL12 (4.80-fold, 95%CI [2.19;5.41] for the difference in means), CCL2 (8.73-fold,
234 95%CI [3.59;11.86] for the difference in means) and OPG (1.75-fold, 95%CI [0.13;1.37]
235 for the difference in means) was increased when prehypertrophic chondrocytes became
236 hypertrophic (Fig. 3I and J). They also tended to overexpressed Rankl mRNA, as
237 compared to prehypertrophic chondrocytes (22.5-fold, 95%CI [-4.32;47.43] for the
238 difference in means) (Fig. 3J). Hypertrophic chondrocytes expressed more Vegf (1.97-fold,
239 95%CI [0.203;1.74] for the difference in means) and Mmp3 mRNAs (22.21-fold, 95%CI
240 [10.29;32.14]) for the difference in means) and less the angiostatic factors Chm1 (-5.99-
241 fold, 95%CI [-0.93;-0.73]) for the difference in means) and Angptl4 than prehypertrophic
242 chondrocytes (-1.65-fold, 95%CI [-0.68;-0.10]) for the difference in means) (Fig. 3H and
243 K). In contrast, neither Tgfb β 1 nor Mpm13, Adamts4 and Adamts5 mRNAs were
244 modulated by the switch from prehypertrophic to hypertrophic differentiation (Fig. 3H and
245 I).

246

247 **Overexpression of IL-34 by hypertrophic chondrocytes**

248 Hypertrophic differentiation of chondrocytes may contribute to OA via the release of
249 factors with autocrine and paracrine tissue remodeling activity. We focused the analysis on
250 ccl, cxcl cytokines/chemokines and ILs, for which expression was upregulated with
251 chondrocyte hypertrophy (Table S2). Seven cytokines/chemokines were overexpressed by
252 hypertrophic chondrocytes, especially in relation to prehypertrophic chondrocytes. Among
253 them, only CXCL12 and IL-34 have their receptors (CXCR4, PTPRZ1 and CSF1R) also
254 upregulated, suggesting that they may act in an autocrine loop (Table S2). Interestingly,
255 *Ptpz1* was the most overexpressed gene during the hypertrophic differentiation of
256 chondrocytes (72.1-fold increased expression compared to iMACs), just after *Mmp3*. In
257 contrast, the levels of *Cxcr7*, the second receptor of CXCL12, were unchanged.

258 Since the involvement of IL-34 in OA is unknown, we next focused our investigation on
259 its expression in OA. Consistent with the microarray analysis, RT-qPCR confirmed the
260 overexpression of Il-34, *Ptpz1* and *Csf1r* in hypertrophic chondrocytes (n=6) (Fig. 4A-C).
261 Both Il-34 and *Ptpz1* mRNA levels increased progressively during the hypertrophic
262 differentiation of chondrocytes, whereas the increase in the expression of *Csf1r* mRNA
263 was observed during the conversion of iMACs to prehypertrophic chondrocytes.
264 Concentrations of IL-34 in both cell supernatants and cell lysates also increased with
265 iMAC hypertrophic differentiation (Fig. 4D and E). Similar results were also observed
266 during the hypertrophic differentiation of human chondrocytes. Their hypertrophic
267 differentiation was associated with a decrease in the mRNA levels *Sox9* and aggrecan, an
268 increase in those of *TNAP* and *MMP-13*, and the presence of areas of calcification within
269 the extracellular matrix (Fig. S3). Human hypertrophic chondrocytes also showed an
270 increase in IL-34 mRNA expression ($P_{adj}=0.011$, 95%CI [0.22;1.17], n=7) and in IL-34

271 concentration in cell supernatant (P_{adj}=0.0013, 95%CI [0.89;2.08], n=6) and cell lysates
272 (P_{adj}=0.0282, 95%CI [0.27;3.17], n=6) (Fig. 4F-H).

273 IL-34 was also released by human OA cartilage, regardless of whether it originated
274 from articular cartilage or from the thin cartilage layer covering osteophytes (Fig. 5A).
275 However, cartilage from osteophytes released higher amounts of IL-34 than OA articular
276 cartilage (P=0.0368, 95%CI [23.43;506.40]). OA tissues also expressed PTPRZ1 and
277 CSF1R (Fig. 5B). Within articular cartilage, positive immunostaining for PTPRZ1 and
278 CSF1R was mainly detected in the chondrocytes of the deeper area of the cartilage or in
279 clusters of chondrocytes, although not all isolated chondrocyte or chondrocyte clusters
280 were positive (Fig. 5B, panels a-f). A more intense immunostaining was observed within
281 the bone and was associated with osteoblasts, osteocytes and cells present in vascular
282 channels, including vessels and mesenchymal stromal cells. A similar positive
283 immunostaining pattern was observed within osteophytes (Fig. 5B, panels g-l).
284 Chondrocytes, osteoblasts, osteocytes and bone marrow cells were positive for both
285 CSF1R and PTPRZ1. In addition, mesenchymal cells of the fibrous tissue, which often
286 covered the osteophyte surface, were also positive for PTPRZ1 immunostaining (Fig. 5B,
287 panels j and k).

288

289 **IL-34 increases the remodeling potential of hypertrophic chondrocytes and** 290 **osteoblasts**

291 We next determined the chondrocyte (n=7) and osteoblast response (n=5) to IL-34. No
292 effect of IL-34 on iMACs and prehypertrophic chondrocytes was observed (data not
293 shown). In contrast, both hypertrophic chondrocytes and osteoblasts were sensitive to IL-
294 34 stimulation. A dose-dependent increase in the mRNA expression of Ccl2, Cxcl12 and

295 Mmp13 by hypertrophic chondrocytes was observed in response to IL-34 (Fig. 6A-C). This
296 was associated with a dose-dependent increase in the release of CCL2 and MMP-13
297 (P=0.0360 and P=0.0489 for CCL2 and MMP-13, respectively) (Fig. 6E-F). No increase in
298 MMP-3 expression and release was observed (Fig. 6D and G). Similarly, IL-34 tended to
299 stimulate the expression of Ccl2 (P=0.0911), Mmp3 (P=0.129), Mmp13 (P=0.141) and
300 Tnf α (P=0.1301), by osteoblasts (Fig. 7A-D). Consistently, IL-34 stimulated the release of
301 CCL2 (P=0.0005), MMP-3 (P=0.0085) and MMP-13 (P=0.0052) by IL-34 in a dose-
302 dependent manner (Fig. 7G-I). IL-34 induced also a dose-dependent decrease in the mRNA
303 expression of both Pedf (P=0.0084) and Ptpz1 (P=0.0917) by osteoblasts (Fig. 7E-F). IL-
304 34 had no observable effect on the mRNA expression of Vegf, Rankl and Csf1r in both
305 hypertrophic chondrocytes and osteoblasts (data not shown).

306

Discussion

307

308

309 OA is characterized at the cellular level by deep phenotypic modifications of cells from
310 the different joint tissues. Notably, there is a hypertrophic differentiation of chondrocytes
311 leading to the accumulation of calcified depots within cartilage ¹⁹ and an advancement of
312 the mineralization front in the deeper part of the cartilage. Hypertrophic differentiation of
313 chondrocytes during OA is thought to play an important role in cartilage disappearance and
314 subchondral bone remodeling ^{20, 21}. Therefore, the identification of molecular factors
315 produced by hypertrophic chondrocytes and involved in cartilage and bone damage in OA
316 could be of therapeutic interest. However, no current model of chondrocyte hypertrophic
317 differentiation is able to lead such investigations. None use articular chondrocytes to obtain
318 hypertrophic chondrocytes able to calcify their matrix. Either they do not provide sufficient
319 quantities of hypertrophic chondrocytes or are based on cell lines rather than primary
320 cultures.

321 Thus, developing such a model that combines all these features represents a real need
322 and a great challenge. Here, we have developed an original model of progressive articular
323 chondrocyte hypertrophic differentiation and identified the recently discovered IL-34 as a
324 factor with putative osteochondral remodeling activity.

325 Our model of chondrocyte hypertrophic differentiation was achieved starting with
326 primary cultures of iMACs. Calcification was only observed after culturing iMACs
327 successively in Medium 1, Medium 2 and then in Medium 3. In addition, only a faint
328 expression of osteocalcin, Osterix and Tnap was observed in the iMAC cultures,
329 suggesting that matrix calcification was not due to contamination of cultures by
330 osteoblasts. Although a transdifferentiation of some chondrocytes into osteoblast-like cells
331 during the culture time cannot be totally ruled out, we consider that the features we

332 observed are attributable to hypertrophic chondrocytes rather than osteoblast-like cells
333 since the expression of Col10a1, a specific marker of hypertrophic chondrocytes, was
334 strongly increased during the culture time.

335 Chondrocyte hypertrophy is generally only determined by the combination of an
336 increased expression of some hypertrophic markers with the decreased expression of
337 chondrocyte markers ²²⁻²⁵. Nevertheless, in addition to these criteria, matrix calcification
338 appears necessary to ascertain that chondrocytes reach hypertrophy. Indeed, chondrocytes
339 cultured in Medium 2 never calcified, although they showed a mRNA expression of
340 hypertrophic markers. The current published culture models of hypertrophic differentiation
341 reaching matrix calcification include primary cultures of chondrocytes isolated from limb
342 buds and growth plate, ATDC5 cells and mesenchymal stem cells (MSCs) ²⁶⁻³⁰. However,
343 neither ATDC5 cells nor MSCs are chondrocytes, and despite the similarities between
344 chondrocytes from growth plates and articular cartilage, they have distinct molecular
345 phenotypes ³¹⁻³³. These models are therefore not suitable for studying hypertrophic
346 articular chondrocytes. Yahara *et al.* also reported matrix calcification with cultures of
347 articular chondrocytes ¹⁷. Pellet cultures of human OA articular chondrocytes expressed
348 hypertrophic markers and showed calcifications ¹⁷ (and this study). However, only sparse
349 calcifications were observed, suggesting that only a subset of chondrocytes reach
350 hypertrophy. Nevertheless, this model appears useful to validate our results obtained with
351 iMACs.

352 In addition to iMACs and hypertrophic chondrocytes, our model provides insight into a
353 third chondrocyte phenotype, which we considered as prehypertrophic chondrocytes
354 considering its gene expression pattern. This chondrocyte population expressed
355 intermediate levels of chondrocyte and hypertrophic markers compared to those expressed
356 in iMACs and hypertrophic chondrocytes, and they did not calcify their matrix. These cells

357 also expressed known prehypertrophic markers, including snorc and osteomodulin^{9, 34},
358 whose expression was stronger than that in both iMACs and hypertrophic chondrocytes.
359 The prehypertrophic phenotype corresponded to a specific population of chondrocytes
360 showing a homogeneous molecular pattern, as revealed by PCA of the microarray results.
361 The whole genome transcriptomic analysis indeed revealed that two phenotypically distinct
362 populations of chondrocytes were obtained from iMACs. We characterized them as
363 prehypertrophic and hypertrophic chondrocytes based on their gene expression pattern and
364 their ability to calcify or not their extracellular matrix. Investigating other features,
365 including the cell shape and the composition and the organization of the extracellular
366 matrix, would be of interest to validate prehypertrophic and hypertrophic states.

367 The presence of several populations of cells with molecularly distinct phenotypes within
368 articular cartilage has recently been described in human OA cartilage^{35, 36}. Ji *et al.*
369 characterized seven different chondrocyte populations, including prehypertrophic and
370 hypertrophic chondrocytes³⁶. Their results suggest that prehypertrophic chondrocytes
371 localized in the deeper part of articular cartilage play an important role in OA progression.
372 Here, we show that prehypertrophic chondrocytes is the most sensitive chondrocyte
373 population to an inflammatory stimulus. Inflammatory stress is a hallmark of OA, and IL-
374 1 β induced a more potent global response by prehypertrophic chondrocytes than by iMACs
375 and hypertrophic chondrocytes. In addition, the prehypertrophic to hypertrophic
376 differentiation of chondrocytes is associated with an increase in the bone remodeling and
377 angiogenic potential of chondrocytes, as evaluated by their molecular pattern. Functional
378 studies will be needed to ascertain the increased potential for tissue remodeling of
379 hypertrophic chondrocytes.

380 The microarray analysis for cytokines/chemokines overexpressed with chondrocyte
381 hypertrophic differentiation and able to act in an autocrine and paracrine manner

382 highlighted the recently discovered IL-34³⁷. RT-qPCR results confirmed this increased
383 mRNA expression, and we also showed that murine and human chondrocytes produced IL-
384 34 at higher rates when chondrocytes were hypertrophic. In OA, hypertrophic
385 chondrocytes are localized within articular cartilage and osteophytes, where they are
386 suspected to play a major role in the pathological remodeling of the osteochondral
387 junctions. Both articular and osteophytic cartilages from OA patients released IL-34. A
388 differential transcriptomic analysis of articular and osteophytic cartilage from paired OA
389 patients revealed a higher expression of genes with functions in terminal chondrocyte
390 differentiation by osteophytic cartilage³⁸. Interestingly, we found that osteophytic cartilage
391 released higher amounts of IL-34 than articular cartilage. In addition, PTRZ1 was among
392 the most upregulated genes in osteophytic cartilage compared to its gene expression in
393 articular cartilage³⁸. Both osteophytic and articular cartilages showed positive
394 immunostaining of PTPRZ1. In particular, OA cartilage PTPRZ1-positive chondrocytes
395 were preferentially located in the deeper zone of joint cartilage or in the chondrocyte
396 clusters, the two areas where hypertrophic chondrocytes are usually found^{7,19}. Osteoblasts
397 and osteocytes of the subchondral bone also express PTPRZ1, as described³⁹, as well as
398 cells present in vascular channels, including vessels and mesenchymal stromal cells. We
399 found a similar expression pattern for CSF1R, whose the expression by bone cells and the
400 increased expression in OA cartilage has been already reported by others^{40,41}.

401 Considering the expression of IL-34 and its receptors by cells of the
402 cartilage/subchondral bone interface, IL-34 may act as a paracrine and autocrine factor on
403 cartilage and bone cells in OA. IL-34 is indeed a known osteoclastogenesis factor^{42,43} and
404 has been reported to stimulate angiogenesis⁴⁴. Its biological activity on both chondrocytes
405 and osteoblasts has never been investigated. Neither iMACs nor prehypertrophic
406 chondrocytes responded to IL-34 stimulation. This may be explained by the differential

407 expression of IL-34 receptors on iMACs, prehypertrophic and hypertrophic chondrocytes.
408 However, the involvement of other molecular partners differentially produced by the
409 phenotypically distinct chondrocytes cannot be excluded as IL-34 displays some of its
410 biological activities independently to Ptpcr1 and Csf1r. It may also bind to other cytokines
411 to form heteromeric cytokines ^{45, 46}. In hypertrophic chondrocytes and osteoblasts, IL-34
412 stimulated the mRNA expression and the release of some tissue remodeling factors,
413 especially the mRNA expression of Cxcl12 in hypertrophic chondrocytes, the secretion of
414 CCL2 and MMP-13 by hypertrophic chondrocytes and osteoblasts and the release of
415 MMP-3 by osteoblasts. Although IL-34 did not stimulate VEGF expression, it may
416 indirectly induce angiogenesis by stimulating the expression of CXCL12 and by inhibiting
417 that of the angiostatic factor PEDF. In addition to its reported direct action on
418 osteoclastogenesis and angiogenesis ⁴²⁻⁴⁴, IL-34 may thus indirectly stimulate these two
419 processes by acting on hypertrophic chondrocytes and osteoblasts. Cartilage-derived IL-34
420 may also explain the positive association between IL-34 concentration in synovial fluid
421 and the radiographic and symptomatic severity of knee OA ⁴⁷. Further studies are needed
422 to explore the specific role of IL-34, especially on human hypertrophic chondrocytes and
423 osteoblasts, and more precisely that of hypertrophic-derived IL-34 in OA.

424 Some limitations emerge from our study. They include the characterization of our
425 model of iMAC hypertrophic differentiation, which would be enriched with the study of
426 the cell shape and the extracellular matrix composition and organization. Functional
427 studies are also needed to ascertain the increased potential for tissue remodeling of
428 hypertrophic chondrocytes and the role of IL-34.

429 To conclude, we have developed a new model of articular chondrocyte hypertrophic
430 differentiation, which allows obtaining three molecularly distinct populations: iMACs,
431 prehypertrophic chondrocytes and matrix calcifying hypertrophic chondrocytes. Our

432 results support the hypothesis that the phenotypic alterations of prehypertrophic
433 chondrocytes in articular cartilage are critical for the loss of cartilage homeostasis observed
434 in OA. The prehypertrophic to hypertrophic differentiation of chondrocytes induced the
435 expression of a subset of genes, which together may favor the pathological remodeling of
436 cartilage and bone, as observed in OA. Notably, the increased production of IL-34 by
437 hypertrophic chondrocytes could act locally on hypertrophic chondrocytes and osteoblasts
438 to indirectly stimulate osteoclastogenesis and angiogenesis. Therefore, according to the
439 tissue remodeling potential of the recently discovered IL-34, further investigations are
440 needed to determine whether IL-34 could be targeted in OA and/or may be used as a
441 synovial biomarker to determine the severity of OA.

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442

443

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450

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451

452

- 453 • Conception and design: MHLP, PP, GRV, FDC, FB, XH
- 454 • Analysis and interpretation of the data: SVE, MLPL, IT, DV, CB, SCG, LG, MHLP,
455 PP, GRV, FDC, FB, XH
- 456 • Drafting of the article: SVE, DV, FB, XH
- 457 • Critical revision of the article for important intellectual content: DV, FB, XH
- 458 • Final approval of the article: SVE, MLPL, IT, DV, AP, DC, CB, SCG, LG, DC, SM,
459 GN, AS, MHLP, PP, GRV, FDC, FB, XH
- 460 • Provision of study materials or patients: AS
- 461 • Statistical expertise: DV
- 462 • Obtaining of funding: FP, MHLP, FDC, FB, XH
- 463 • Collection and assembly of data: SVE, MLPL, IT, AP, DC, CB, DC, SM, GN, AS, XH

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472

473

Competing interests

474 None.

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475

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- 613
- 614

Figure legends

615

616 **Figure 1. Hypertrophic chondrocyte differentiation markers assessed in iMACs**
617 **differentiated into prehypertrophic and hypertrophic chondrocytes.**

618 **A)** Model of progressive hypertrophic differentiation of iMACs. iMACs isolated from the
619 femoral heads and the knees of newborn mice were cultured for seven days in Medium 1.
620 Confluent iMACs were then cultured for 28 days in Medium 2 to obtain prehypertrophic
621 chondrocytes and for an additional 42 days for prehypertrophic to hypertrophic phenotype
622 changes. **B–N)** Gene expression pattern of articular chondrocyte markers (**B–E**),
623 prehypertrophic markers (**F–H**) and hypertrophic markers (**I–N**) in iMACs, prehypertrophic
624 chondrocytes and hypertrophic chondrocytes from 6 independent cell cultures. The results
625 are expressed as fold expression compared to those of iMACs, whose mRNA expression
626 was set to 1 for each culture. **O)** Measurement of alkaline phosphatase activity associated
627 with iMACs, prehypertrophic chondrocytes and hypertrophic chondrocytes (n=8). Lower
628 panels, representative photomicrographs of the cytochemical determination of phosphatase
629 alkaline activity in iMACs, prehypertrophic chondrocytes and hypertrophic chondrocytes.
630 **P)** Quantification of chondrocyte culture mineralization by alizarin red staining (n=12).
631 Lower panels, representative photomicrographs of alizarin red staining of iMACs,
632 prehypertrophic chondrocytes and hypertrophic chondrocytes. Only hypertrophic
633 chondrocytes showed positive alizarin red staining. Bars indicate the mean expression
634 levels.

635

636

637

638 **Figure 2. Whole-genome transcriptomic characterization across iMAC differentiation**
639 **processes into hypertrophic chondrocytes.**

640 Results from differential analysis of gene expression in the chondrocytes. Signatures of
641 differentially expressed genes (DEG) identified between **A)** hypertrophic vs articular
642 chondrocytes (n=8 in each group), **B)** prehypertrophic vs articular (n=8 in each group), **C)**
643 and hypertrophic vs prehypertrophic chondrocytes (n=8 in each group). A Venn diagram
644 compares the signatures obtained. Each comparison is illustrated by a hierarchical
645 clustering (correlation distance on genes and Euclidean distance on samples). Volcano
646 plots present the log₂-fold changes and the significance of each gene for the 3
647 comparisons. Gene and probe numbers differ due to repeated spotting and/or missing
648 annotations.

649

650 **Figure 3. Loss of the prehypertrophic phenotype shifts chondrocytes towards an OA-**
651 **inducing phenotype.**

652 **A-G)** iMACs, prehypertrophic chondrocytes and hypertrophic chondrocytes from 6
653 independent cell cultures were stimulated by IL-1 β (1 ng/mL) for 24 hours, and the mRNA
654 expression of Col2a1 (**A**), Acan (**B**), Il-6 (**C**), Mmp13 (**D**), Vegf (**E**), Tsp1 (**F**) and Chm1
655 (**G**) was determined. **H-K)** Phenotypic transition of prehypertrophic to hypertrophic
656 chondrocytes induces the expression of factors involved in OA. Prehypertrophic and
657 hypertrophic chondrocytes from 6 to 12 independent cell cultures were assessed for the
658 mRNA expression of matrix proteases (**H**), osteoblast (**I**) and osteoclast activity (**J**) and
659 angiogenic/angiostatic factors (**K**). Data are expressed as fold expression compared to
660 those in unstimulated control cells, whose mRNA expression was set to 1 for each culture

661 (A-G), or to that in prehypertrophic chondrocytes (H-K). Bars indicate the mean
662 expression levels.

663

664 **Figure 4. Increased expression of IL-34 and IL-34 receptors with chondrocyte**
665 **hypertrophic differentiation.**

666 A-E) mRNA expression of Il-34 (A), Pptrz1 (B) and Csf1r (C) in iMACs, prehypertrophic
667 chondrocytes and hypertrophic chondrocytes (n = 6) was determined. C-D) IL-34 protein
668 levels were quantified by ELISA in cell conditioned medium (n = 7) (D) and cell lysates (n
669 = 6) (E) of iMACs, prehypertrophic chondrocytes and hypertrophic chondrocytes. F-H)
670 The IL-34 mRNA expression (n = 7) (F) and protein levels (n = 6) in chondrocyte
671 conditioned medium (G) and cell lysates (H) were determined in human control and
672 hypertrophic chondrocytes.

673

674 **Figure 5. Expression of IL-34 and IL-34 receptors in OA.**

675 A) IL-34 secreted by articular (n = 14 different donors) and osteophyte cartilages (n = 6
676 different donors) from OA patients was measured in tissue conditioned media by ELISA.
677 B) Paraffin sections (5 µm) of OA cartilage bone interface (a-f) and osteophytes (g-l) (n =
678 5 to 8 different donors) were stained for CSF1R (a, b, g and h), PTPRZ1 (d, e, j and k) or
679 with irrelevant antibodies as negative controls (c, f, i and l). CSF1- and PTPRZ1-positive
680 staining are observed at the osteochondral junction (a, b, d and e). Chondrocytes near the
681 tidemark express both the IL-34 receptors CSF1R and PTPRZ1. Within the bone, CSF1R-
682 and PTPRZ1-positive staining is associated with osteoblasts and vascular channels. Similar
683 staining was observed for both CSF1R and PTPRZ1 within osteophytes (g, h, j and k).
684 Chondrocytes, osteoblasts, osteocytes and bone marrow cells showed positive staining. In

685 addition, mesenchymal cells of the fibrous tissue at the osteophyte surface were positive
686 for PTPRZ1. Panels b, e, h and k show higher magnification views of the delimited areas
687 of panels a, d, g and j, respectively. Cartilage and bone are delimited by dotted lines. Bo:
688 bone, Cart: cartilage, CC: calcified cartilage. Bars = 200 μm (a, d, g and j) or 50 μm (b, c,
689 e, f, h, i, k and l).

690

691 **Figure 6. Increased remodeling potential of hypertrophic chondrocytes in response to**
692 **IL-34.**

693 Hypertrophic chondrocytes (n=7) were stimulated by increasing concentrations of IL-34
694 before RT-qPCR analysis of the mRNA expression of Cxcl12 (A), Ccl2 (B), Mmp3 (C)
695 and Mmp13 (D). The release of CCL2 (E), MMP-3 (F) and MMP-13 (G) into cell
696 conditioned medium in response to increased concentrations of IL-34 was measured by
697 ELISAs.

698

699 **Figure 7. Increased remodeling potential of osteoblasts in response to IL-34.**

700 Osteoblasts (n=5) were stimulated by increasing concentrations of IL-34 before RT-qPCR
701 analysis of the mRNA expression of Cxcl12 Ccl2 (A), Mmp3 (B), Mmp13 (C), Tnf α (D),
702 Pedf (E) and Ptpz1 (F). The release of CCL2 (G), MMP-3 (H) and MMP-13 (J) into cell
703 conditioned medium in response to increased concentrations of IL-34 was measured by
704 ELISAs.

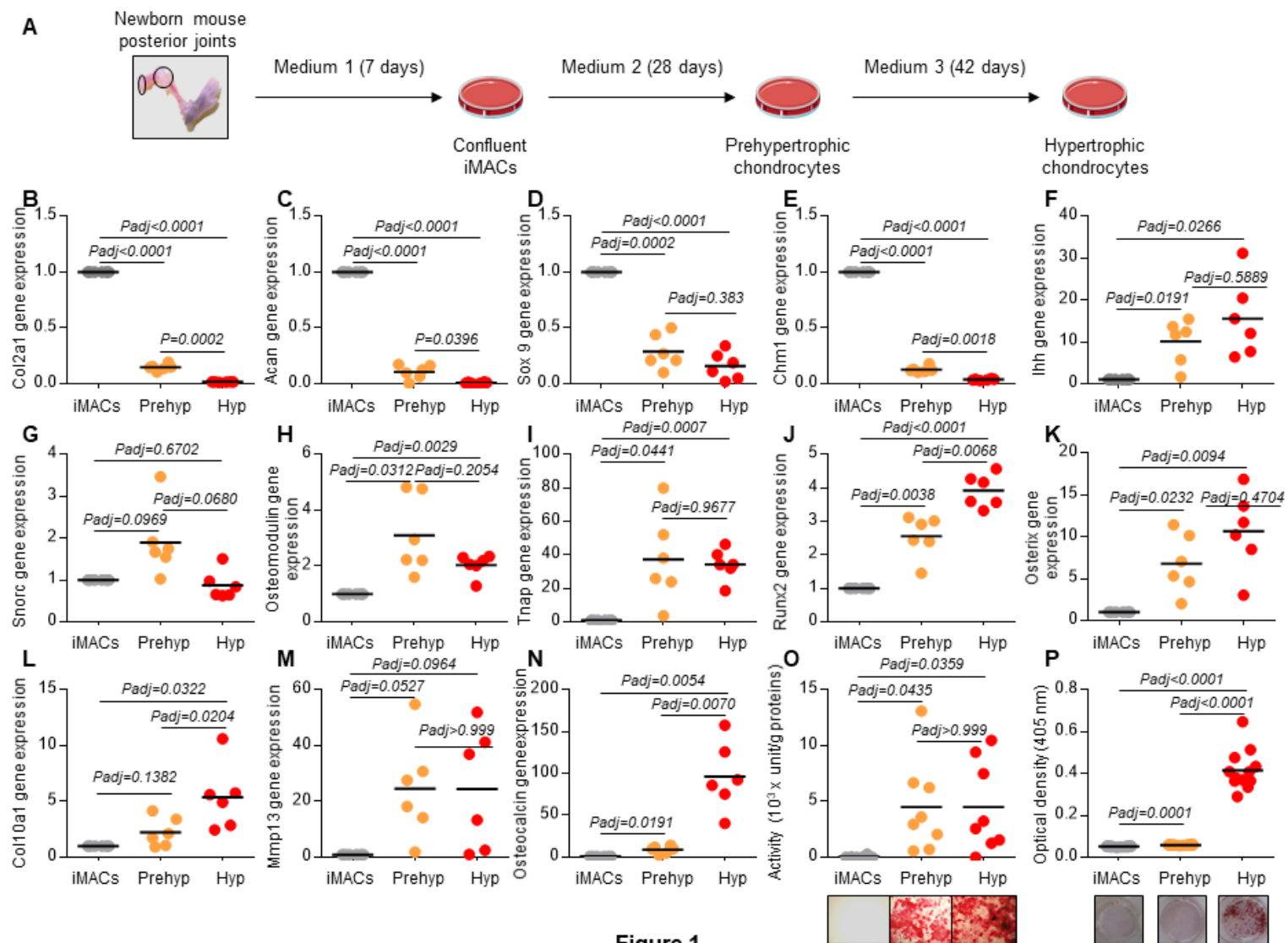


Figure 1

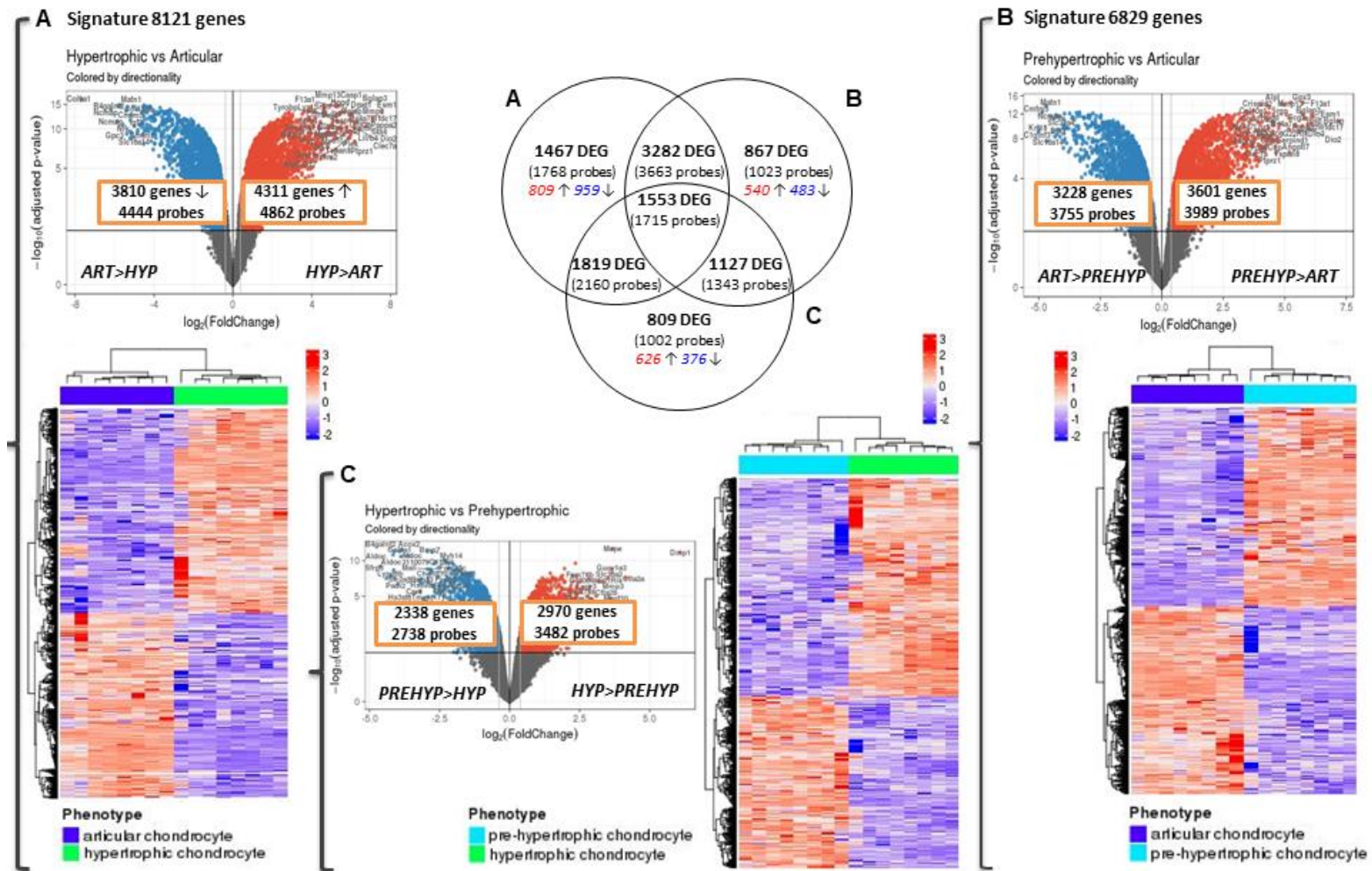


Figure 2

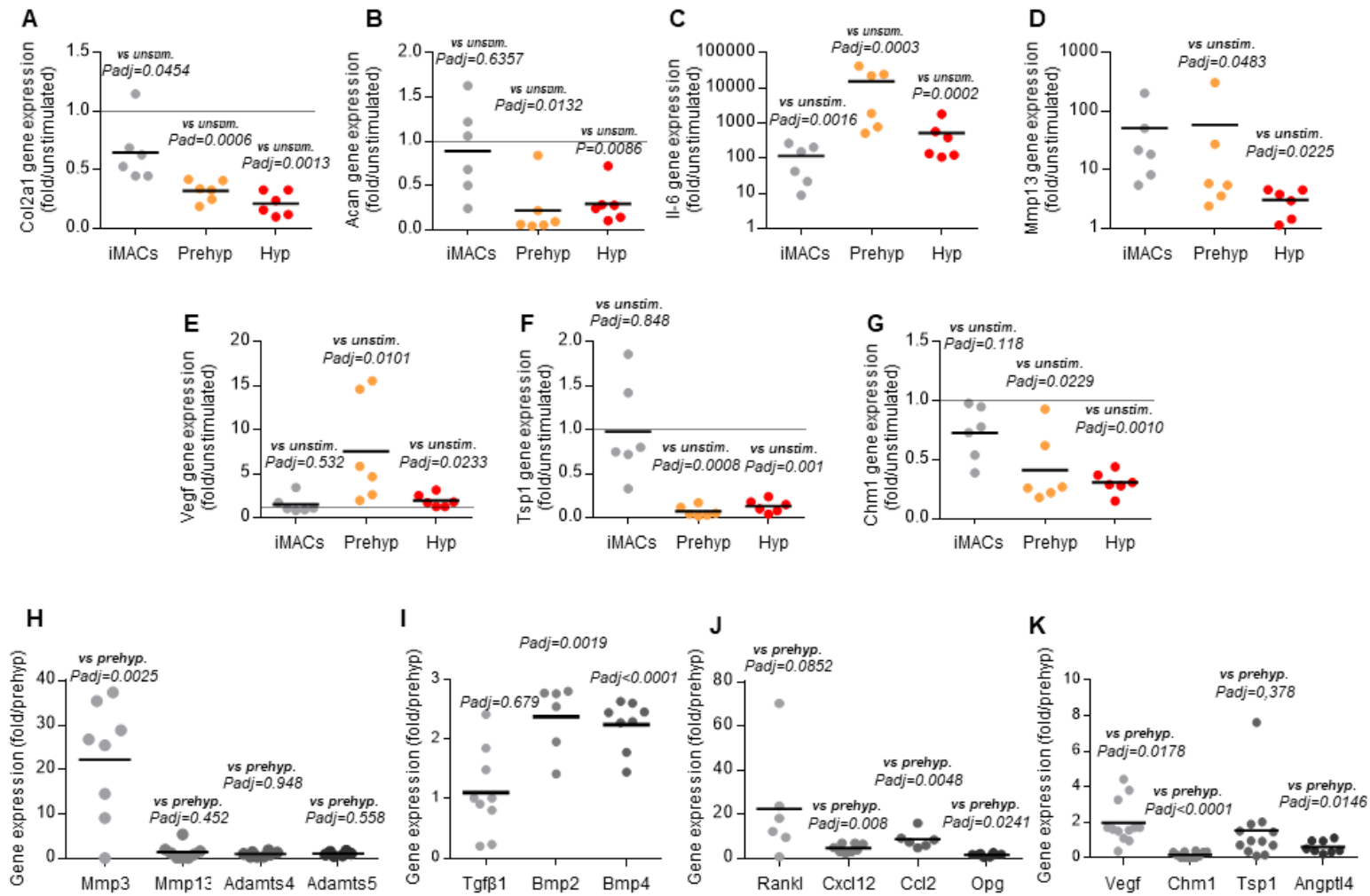


Figure 3

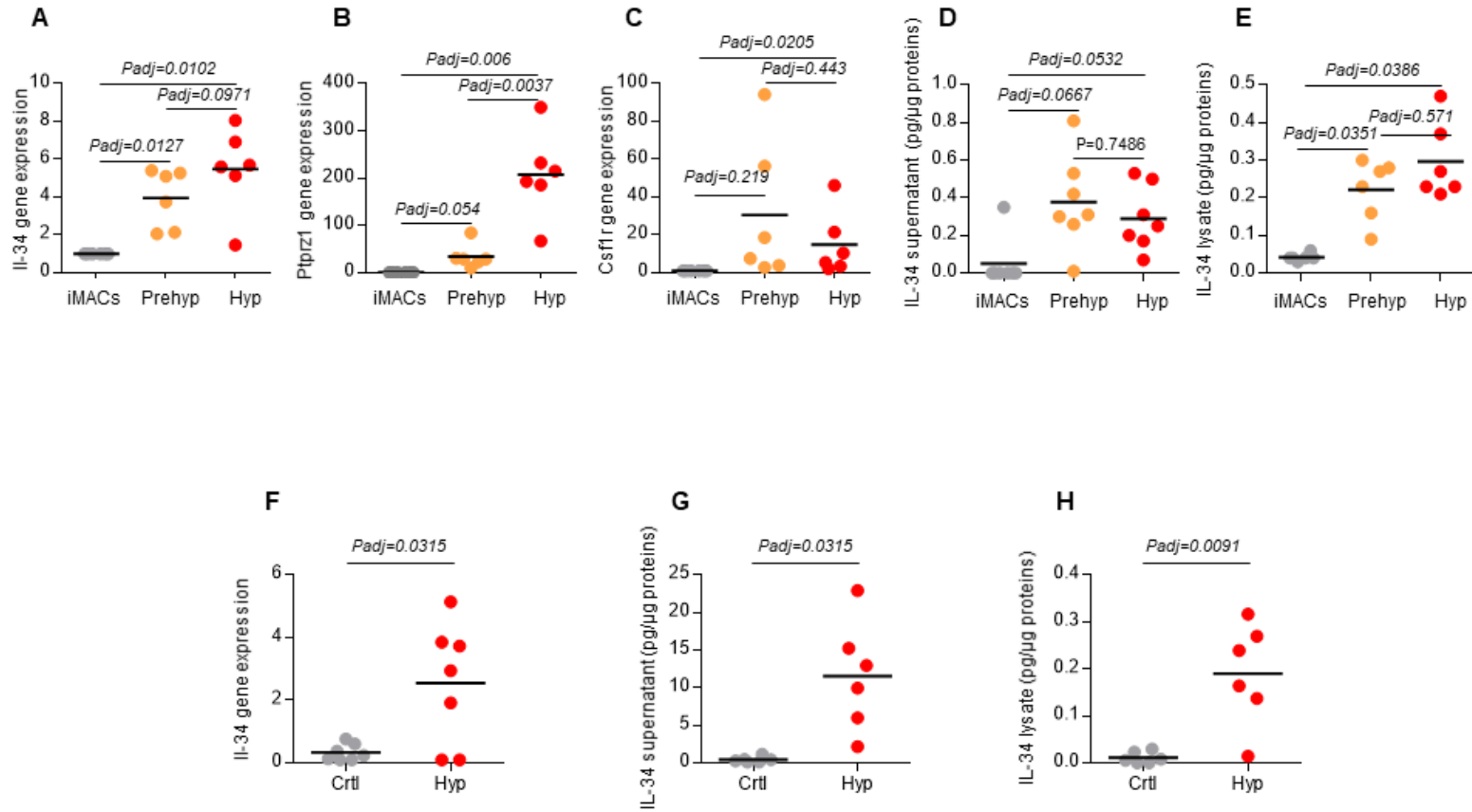


Figure 4

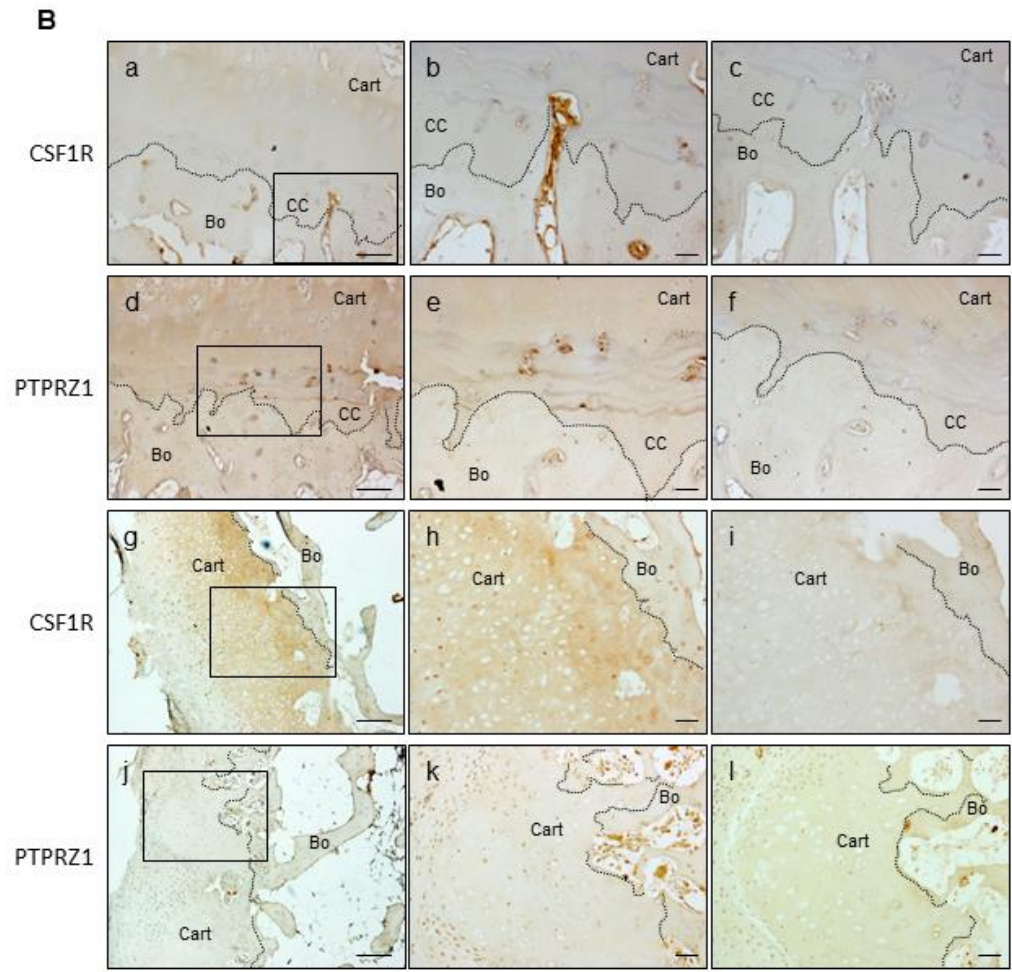
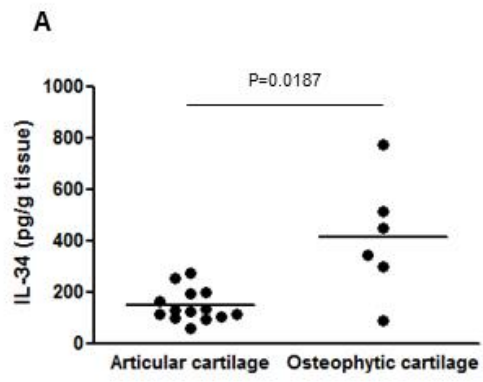


Figure 5

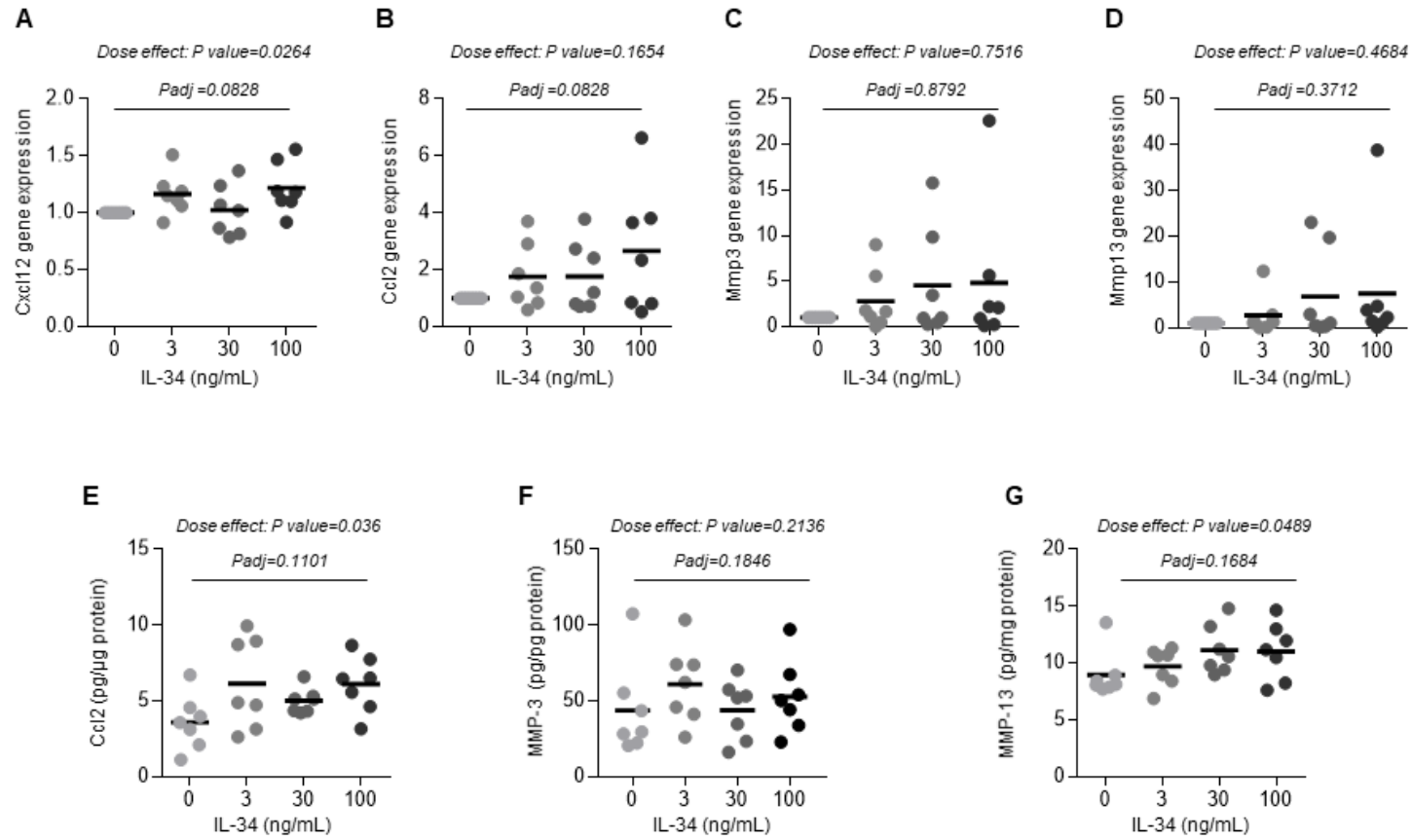


Figure 6

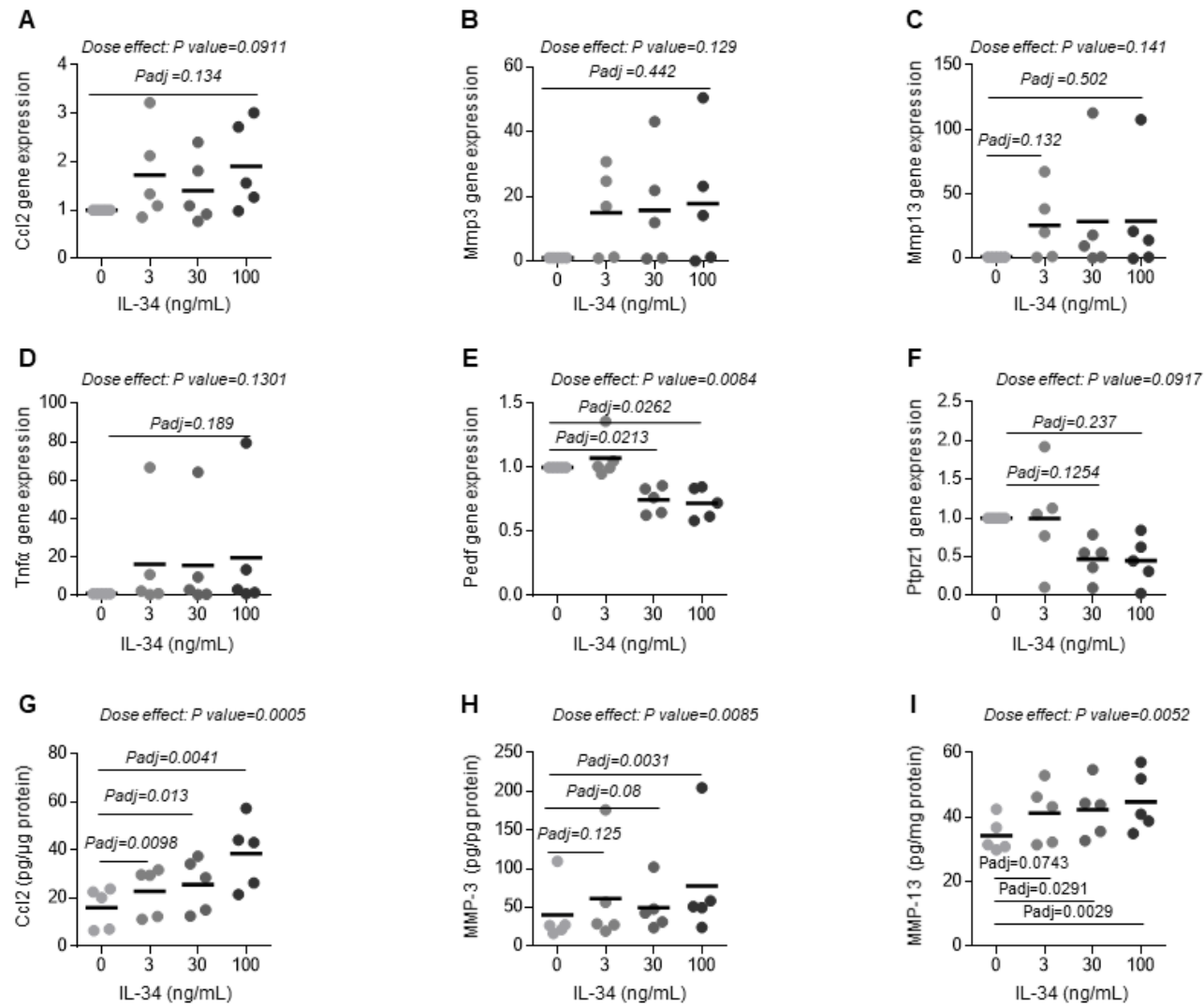


Figure 7

713 **Materials and methods**

714

715 **Collection of osteoarthritis human cartilage and subchondral bone**

716 Human OA knee explants were obtained from 33 patients undergoing total knee joint
717 replacement surgery for OA in the Department of Orthopedic Surgery and Traumatology
718 of Saint-Antoine Hospital and in the Maussins-Nollet Clinic (Paris, France). Informed
719 consent was obtained from each patient on the day before the arthroplasty. Experiments
720 using human samples have been approved by two French Institutional Review Boards
721 (Comité de Protection des Personnes Ile de France V; Comité Consultatif sur le Traitement
722 de l'information en Matière de Recherche). The mean age of patients was 69.6 ± 11.2 years
723 and 78.8% were women. The mean BMI was 30.1 ± 4.5 kg/m².

724 Tissue pieces from the middle part of the medial and lateral tibial plateaus and femoral
725 condyles were preserved for histological analysis. The remaining cartilage from each joint
726 compartment was separated from the underlining bone and cut into small pieces (1 mm³)
727 before a 24-hour incubation at 37°C in RPMI culture medium supplemented with 100
728 U/mL penicillin, 100 µg/mL streptomycin and 4 mM glutamine, as described ¹.
729 Conditioned media was then separated from tissues and centrifuged at 3000 g for 5 min to
730 remove the debris. Conditioned media and tissues were stored at -80°C for further analysis.
731 Alternatively, chondrocytes were isolated for cell culture experiments.

732

733 **Immunohistochemistry**

734 Tissue samples were fixed in 3.7% paraformaldehyde for 2 days and decalcified in a
735 solution of 14% ethylenediaminetetraacetic acid in distilled water (pH 7.4) for 4 to 6 weeks
736 at 4°C. Samples were embedded in paraffin wax and serially sectioned (5 µm).

737 Immunohistochemistry was performed with a mouse monoclonal antibody against
738 PTPRZ1 (clone 12/RPTPb, BD Transduction Laboratories; dilution 1:50) and a rabbit
739 polyclonal antibody against CSF-1R (H-300, Santa Cruz Biotechnology; dilution 1:50) as
740 the primary antibodies. Enzyme-induced antigen retrieval was performed as follows: 0.2
741 mg/mL hyaluronidase in phosphate-buffered saline (PBS) (pH 5.5) for 10 min at 37°C and
742 then 0.1 mg/mL pronase in PBS (pH 7.4) for 20 min at 37°C for CD34, OC and PTP- ζ , and
743 2 mg/mL hyaluronidase in PBS (pH 5.5) for 10 min at 37°C and 1 mg/mL pronase in PBS
744 (pH 7.4) for 20 min at 37°C for CSF-1R. The R.T.U. Vectastain kit (Vector) was used for
745 detection, followed by counterstaining with Mayer's hematoxylin. Irrelevant control
746 antibodies (Dako) were incubated at the same concentration to assess nonspecific staining.
747 Preparations were mounted in Eukitt medium. Digital images of magnification views
748 (10X) of whole-tissue sections were captured by using an Olympus SC50 camera on an
749 Olympus IX83 microscope.

750

751 **Primary culture of murine articular chondrocytes and differentiation into** 752 **hypertrophic chondrocytes**

753 Immature articular chondrocytes (iMACs) were isolated from the femoral heads and
754 knees of 5-6-day-old newborn C57BL/6 mice (Janvier labs) and cultured in DMEM culture
755 medium (1 g/L glucose) supplemented with fetal calf serum (10%), penicillin (100 U/mL),
756 streptomycin (100 μ g/mL) and L-glutamine (4 mM)) (Medium 1) for 7 days, as described
757 ^{2, 3}. For each independent cell culture, iMACs were pooled from 5 to 7 newborn
758 littermates. After the cells were seeded in the culture plates, the culture wells containing
759 the cells were randomly allocated to the different experimental groups. Prehypertrophic
760 chondrocytes were obtained by culturing chondrocytes for an additional 28 days in culture
761 medium 2 consisting of DMEM/HAM-F12 medium supplemented with fetal calf serum

762 (5%), penicillin (100 U/mL), streptomycin (100 µg/mL) and L-glutamine (4 mM), ascorbic
763 acid (40 µg/mL, Sigma-Aldrich), insulin-transferrin-sodium selenite (1%, Sigma-Aldrich)
764 and triiodo-L-thyronine (50 ng/mL, Sigma-Aldrich) (Medium 2). Prehypertrophic
765 chondrocytes were further cultured for 42 days in the latter medium supplemented with β-
766 glycerophosphate (10 mM, Sigma-Aldrich), retinoic acid (100 nM, Sigma-Aldrich) and
767 1α,25-dihydroxyvitamin D₃ (10 nM, Sigma-Aldrich) (Medium 3) to obtain hypertrophic
768 chondrocytes. All cultures were performed at 37°C in a humidified atmosphere of 5%
769 CO₂/95% air with the exception of the prehypertrophic to hypertrophic differentiation step,
770 which was performed at 37°C in a humidified atmosphere of 3% CO₂/95% air. At the end
771 of the culture, chondrocytes were washed 2 times with PBS and incubated in DMEM (1
772 g/L glucose) without serum for 4 hours. Chondrocytes were then washed with PBS and
773 further incubated in DMEM without serum for 24 hours. Conditioned media were then
774 kept, centrifuged and stored at -20°C. Cells were either fixed in 3.7% paraformaldehyde
775 (PFA) for cytological analysis or used for mRNA or protein extraction.

776

777 **Primary culture of murine osteoblasts**

778 Osteoblasts were isolated from the calvaria of 5- to 6-day-old newborn C57BL/6 mice
779 (Janvier labs), as previously described ⁴. For each independent cell culture, osteoblasts
780 were pooled from 5 to 7 newborn littermates. After the cells were seeded in the culture
781 plates, the culture wells containing the cells were randomly allocated to the different
782 experimental groups. Briefly, osteoblasts were cultured for 21 days in DMEM/HAM-F12
783 supplemented with fetal calf serum (10%), penicillin (100 U/mL), streptomycin (100
784 µg/mL), L-glutamine (4 mM), and ascorbic acid (50 µg/mL, Sigma-Aldrich). After 10 days
785 of culture, β-glycerophosphate (5 mM, Sigma-Aldrich) was added to the culture medium.
786 At the end of the culture, osteoblasts were washed 2 times with PBS and incubated in

787 DMEM (1 g/L glucose) without serum for 4 hours. Osteoblasts were then washed with
788 PBS and further incubated in DMEM without serum for 24 hours. Conditioned media were
789 then kept, centrifuged and stored at -20°C. Cells are used for mRNA extraction.

790 All experiments with murine chondrocytes and osteoblasts were performed according to
791 the protocols approved by French and European ethics committees (Comité d’Ethique en
792 Expérimentation Animale n°5 Charles Darwin de la Région Ile de France).

793

794 **Stimulation of primary cultures of murine chondrocytes and osteoblasts**

795 Before treatment, iMACs, prehypertrophic chondrocytes, hypertrophic chondrocytes or
796 osteoblasts were cultured for 24 hours in serum-free DMEM (1 g/L glucose) containing
797 penicillin (100 U/mL) and streptomycin (100 µg/mL). Cells were stimulated in the same
798 medium by human recombinant IL-1β (1 ng/mL) (PeproTech) or murine recombinant IL-
799 34 (PeproTech) at increasing concentrations (3, 30 and 100 ng/mL). After 24 hours of
800 stimulation, the supernatants and total RNA were kept and stored at -80°C.

801

802 **Primary culture of human articular chondrocytes and differentiation into** 803 **hypertrophic chondrocytes**

804 Human chondrocytes were isolated from the less damaged areas of OA cartilage from
805 patients who underwent total knee arthroplasty. The hypertrophic differentiation of human
806 articular chondrocytes was performed according to Yahara *et al.*⁵. A total of 10⁶
807 chondrocytes were seeded into 10-cm dishes and cultured for 7 days in chondrogenic
808 medium, which was composed of DMEM (4.5 g/L glucose), fetal calf serum (10%), L-
809 glutamine (4 mM), penicillin (100 U/mL), streptomycin (100 µg/mL), insulin-transferrin-
810 sodium selenite solution (1%, Sigma-Aldrich), recombinant human transforming growth

811 factor-beta 1 (TGF- β_1) (10 ng/mL, PeproTech), dexamethasone (100 nM, Sigma-Aldrich),
812 sodium pyruvate (1 mM, Sigma-Aldrich) and L(+)-ascorbic acid (50 μ g/mL, Sigma-
813 Aldrich). Then, $5 \cdot 10^5$ cells were transferred into 15-mL polypropylene tubes, pelleted by
814 centrifugation for 10 min at 500 g and cultured in the same medium for 3 days. Pellets
815 were transferred into 24-well culture plates and cultured for 8 weeks in the same medium.
816 The medium was replaced with DMEM (4.5 g/L glucose), 1% fetal calf serum, L-
817 glutamine (4 mM), penicillin (100 U/mL), streptomycin (100 μ g/mL), 1% insulin-
818 transferrin-sodium selenite (Sigma-Aldrich), recombinant human bone morphogenetic
819 protein-2 (BMP-2) (10 ng/mL, PeproTech), recombinant human growth differentiation
820 factor-5 (GDF-5) (10 ng/mL, PeproTech), triiodo-L-thyronine (1 μ M, Sigma Aldrich),
821 dexamethasone (10 nM, Sigma-Aldrich), L(+)-ascorbic acid (50 μ g/mL, Sigma-Aldrich)
822 and β -glycerophosphate (10 mM, Sigma-Aldrich) for 2 additional weeks. At the end of the
823 culture, chondrocytes were washed 2 times with PBS and incubated in DMEM (1 g/L
824 glucose) without serum for 4 hours. Pellets were then washed with PBS and further
825 incubated in DMEM without serum for 24 hours. Conditioned media were then kept,
826 centrifuged and stored at -20°C . Cell pellets were lysed for mRNA or protein extraction.
827 Some pellets were fixed in 3.7% PFA and embedded in paraffin. The presence of
828 calcification was revealed by von Kossa staining on microsections (5 μ m).

829

830 **RNA extraction, reverse transcription and quantitative real-time PCR**

831 Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Courtaboeuf, France),
832 according to the manufacturer's instructions, and the concentrations were determined using
833 a spectrophotometer (Eppendorf, Le Pecq, France). The RNA integrity was assessed based
834 on the 28S/18S ribosomal RNA ratio using the RNA 6000 Nano Lab-On-Chip with an
835 Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). All RNA samples

836 included in this study had an RIN >8.3. RNA (500 ng) and were reverse transcribed (RT)
837 using an Omniscript RT Kit (Qiagen, Courtaboeuf, France). The mRNA expression of
838 genes of interest was analyzed by quantitative real-time PCR using the Light Cycler 480
839 (Roche Diagnostics, Meylan, France) in a 12 μ L final volume reaction using specific
840 primers (10 μ M) (see Supplementary Tables 3 and 4) and GoTaq® qPCR Master Mix
841 (Promega Corp., Madison, WI, USA). The PCR amplification conditions were as follows:
842 initial denaturation for 5 min at 95°C followed by 40 cycles consisting of 10 s at 95°C, 15 s
843 at 60°C and 10 s at 72°C. For each PCR experiment, the cDNAs were run in duplicate in
844 parallel with serial dilutions of a cDNA mixture tested for each primer pair to generate a
845 standard linear curve, which was used to estimate the amplification efficiency. The relative
846 mRNA expression for all genes analyzed was normalized to that of hypoxanthine guanine
847 phosphoribosyl transferase (*Hprt*) or RNA ribosomal 18S (18S), used as internal reference
848 genes for mouse and human genes, respectively, and the expression was determined using
849 the $2^{-\Delta\Delta C_t}$ method. For the selection of samples before microarray analysis, normalization
850 of qPCR data was realized according the gold standard proposed by Vandesompele *et al.*,
851 2002⁶ implemented in GeNorm software that further evolved and was integrated in qBase
852 Plus software. We assessed the expression of 7 housekeeping genes: *Tbp*, *Hmbs*, *Actb*,
853 *Ppia*, *Gusb*, *RPS18*, *GAPDH*, *RPL30* that were confirmed to be robust reference genes in
854 our laboratory in multiple experiments. Thus, we compute M-values to identify the genes
855 that covary the most between samples that are the most likely to enter in the computation
856 of a normalizer. Normalizer is the geometric mean of most covarying genes that is
857 subtracted to the value of C_t of the sample. Thus, we obtain $-dC_t$ (compared to mean of
858 invariant selected housekeeping genes using qBase+) and Fold Changes [$2^{-\Delta\Delta C_t}$] (each
859 $-dC_t$ sample is compared to $-dC_t$ mean of iMACs).

860

861 **Microarray analysis**

862 To select a sufficient design for microarray analysis, we evaluated among 12
863 independent cultures the most representative cultures of each differentiation group,
864 according to the gene expression of markers of each chondrocyte phenotype, and set up a
865 method for this purpose. Eight cultures were determined to be optimal to ensure sufficient
866 power to avoid unnecessary mouse sacrifices. Score-based selection determined that 7
867 cultures needed to be selected for microarray analyses (Fig. S2A). Combining the method
868 with the best PCA contributions to the principal component 1 (PC1) group allowed us to
869 finally select the 8 cultures among the 12 needed for the microarray experiments that were
870 subsequently performed.

871 mRNA expression profiling was performed on 8 samples per group using SurePrint G3
872 Mouse Gene Expression v2 8x60K Microarray (G4852B, Agilent Technologies) and
873 SurePrint Mouse miRNA Microarray Kit v21 8x60K (G4859C, Agilent Technologies) at
874 the Servier Research Institute (Croissy-Sur-Seine, France). Labeling reactions and
875 hybridizations were randomized according to the chondrocyte differentiation status. For
876 mRNA profiling, probe labeling and 60 mer-oligonucleotide microarray hybridization were
877 performed according to the manufacturer's instructions ⁷. Starting with 100 ng of purified
878 total RNA, cDNA synthesis and *in vitro* transcription were carried out using an Agilent
879 LowInput QuickAmp Labeling Kit (Agilent Technologies, Palo Alto, CA) in the presence
880 of Cyanine3- and Cyanine5-CTP. The fluorescently amplified cRNAs were purified with
881 an RNeasy column (Qiagen, France) and assessed for quantification and incorporation rate
882 using a NanoDrop Spectrophotometer (Cyanine-3-CTP at 550 nm and Cyanine-5-CTP at
883 650 nm). Probe length was controlled using the Agilent 2100 Bioanalyzer (Agilent
884 Technologies, Palo Alto, CA). The two labeled cRNA probes (825 ng each), Cy5-
885 chondrocyte subtypes and Cy3-reference sample (pool of control DMEM RNAs), were

886 pooled and hybridized to SurePrint G3 Mouse Gene Expression v2 8x60K Microarray
887 (G4852B) at 65°C for 17 hours. The reference sample corresponded to a pool of the 8
888 control DMEM conditions, each contributing at an equal amount to this pool. Slides were
889 washed at room temperature in Gene Expression Wash Buffer 1 (5188-5325, Agilent
890 Technologies) for 1 min, then at 37°C in Gene Expression Wash Buffer 2 (5188-5326,
891 Agilent Technologies) for 1 min and finally in acetonitrile for 30 s.

892 An Agilent scanner and Feature Extraction 11.5.1.1 software (Agilent Technologies)
893 were used to obtain the raw microarray data for both analyses.

894

895 **Protein extraction and protein assay**

896 Murine chondrocytes were lysed in Tris HCl (10 mM) pH 7.5, MgCl₂ (0.5 mM) and
897 Triton X-100 (0.1%), as described by Kirsch *et al.*⁸. Cell homogenates were centrifuged at
898 10000 g for 10 min at 4°C. Supernatants were kept for analysis.

899 Human chondrocytes in pellets were lysed in RIPA buffer containing Tris HCl (50 mM;
900 pH 8), NaCl (150 mM), EDTA (2 mM), NP-40 (1%), sodium deoxycholate (0.5%) and
901 sodium dodecyl sulfate (SDS) (0.1%), supplemented with protease inhibitor cocktail
902 cOmplete™ 1X (Roche).

903 Protein concentrations in cell-conditioned media were determined using the Bradford
904 assay (Bio-Rad, Hercules, CA, USA). Protein concentrations in cell lysates were
905 determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific),
906 according to the manufacturer's instructions.

907

908

909

910

911 **Alkaline phosphatase activity assay**

912 Alkaline phosphatase enzymatic activity was measured in murine chondrocyte cell
913 lysates using the Colorimetric Alkaline Phosphatase Assay Kit, Yellow Color
914 (FluoroProbes, Interchim, Montluçon, France).

915

916 **ELISA assessment of the IL-34, MMP-3, MMP-13 and MCP-1 levels**

917 Commercially available ELISA kits were used to determine the concentrations of mouse
918 MMP-3 (R&D Systems), MMP-13 (Euromedex), MCP-1 (R&D Systems), IL-34 (R&D
919 Systems), human IL-34 (R&D Systems).

920

921 **Cytology**

922 The alkaline phosphatase activity was visualized using Naphthol AS-MX phosphate (20
923 mg/mL, Sigma-Aldrich) and Fast Red TR (1 mg/mL, Sigma-Aldrich) in Tris-HCl buffer
924 (36 mM; pH 8.3).

925 The quantification of cell calcification was performed as described ⁹. After alizarin red
926 staining, cells were scraped in 10% (v/v) acetic acid. The solution was heated at 85°C for
927 10 min, transferred to ice for 5 min and centrifuged at 20000 g for 15 min. Then, 250 µL of
928 the supernatant was mixed with 100 µL of 10% (v/v) ammonium hydroxide, and the
929 optical density was measured in triplicate at 405 nm.

930

931 **Statistical analysis**

932 We determined sample size before conducting experiments. We performed three sets of
933 simulations using R or using StatMate (v.2.00) assuming that we will face a beta SD of 0.5,
934 1 or 2 and willing a power of 80%. We found the best tradeoff to us considering number of

935 experimental units, lowest beta estimable but also availability of samples and feasibility of
936 experiences to ensure the less technical biases possible. Integrating all the parameters that
937 have to be taken into account before conducting the experiments we determine that a
938 minimum of 5-6 samples has to be assessed in a single experiment. In an optimistic
939 scenario with low variability we would be able to assess a mean difference of ~0.5 in 80%
940 of the cases. To face additional variability, when it was possible we added more
941 experimental. Some qPCR analysis had even more power since 12 observations were
942 available.

943 For microarrays sample size estimation the method proposed by Pawitan *et al.*¹⁰ - OCS
944 plus R package- was used to estimate the number of samples needed for microarrays
945 analysis. This methodology suggested an optimal number of n=8 samples per group (paired
946 design). For obvious reasons of cost and experience feasibility only 8 samples per group
947 were processed in the microarrays giving the sufficient power to properly detect the
948 expected amount of differentially expressed genes.

949 For further analyses, we used parametric tests after log(x) transformation: normality on
950 markers was verified using the D'Agostino and Pearson and Shapiro-Wilk tests. For
951 biological interpretability, we plotted the untransformed datasets on graphs.

952 The qPCR normalized gene expression levels of hypertrophic markers, alkaline
953 phosphatase activity, cell calcification degrees and release of proteins in media were
954 compared between the iMAC, prehypertrophic and hypertrophic cells using repeated
955 measures one-way ANOVA. Heteroscedasticity was taken into account with the Geisser-
956 Greenhouse correction. Corrections for multiple comparisons were realized using the
957 Tukey range test when contrasting all groups in mouse chondrocytes. For the IL-1 β
958 stimulation study, we used Dunnett's post hoc test when comparing groups of interest with
959 an additional unstimulated group. To analyze the cell response to IL-34, we also used this

960 model and this post hoc testing strategy to respectively determine the global dose response
961 effect (dose p-value in the model) and adjusted p-value for comparison with the
962 unstimulated group (0 ng/ml).

963 For prehypertrophic to hypertrophic chondrocyte gene expression comparison in mice
964 as well as gene or protein comparison in OA patients, paired t-tests were used to compare
965 observations from the two groups of interest (*e.g.*, hypertrophic vs. control or
966 prehypertrophic conditions). For the comparison of protein levels of IL-34 in human
967 articular cartilage vs. osteophytic cartilage, an unpaired t-test was performed with Welch
968 correction for unequal variance. The analyses mentioned above were performed using
969 GraphPad Prism8 (GraphPad Software Inc., San Diego, CA, USA). Values of FC, 95%CI
970 and P-values for all experiments are available in the appendix.

971 For whole-genome transcriptomic analysis, litter selection was performed to ensure
972 sufficient power with minimum mouse sacrifice. N=8 mice was determined to be the
973 optimal number. Based on previous RT-qPCR data obtained, we calculated a score= sum
974 of delta ($|\Delta Ct|$) between extreme phenotypes (hypertrophic and articular) for each litter
975 to select the most variant observations. The litter was selected if they displayed a
976 $score(i) > \text{mean}(\text{scores})$.

977 Graphical explorations on targeted genes and whole-genome data, such as two-
978 dimensional PCA and double hierarchical clustering, were performed with the package
979 “mixOmics” (package “mixOmics” in R ¹¹) under R program writer software
980 (<http://www.r-project.org/>)¹² to evaluate whether clusters appear between cells and if they
981 were associated with differentiation groups.

982 Raw microarray data were analyzed with Arraystudio Omicsoft version 10.0.1.112
983 (QIAGEN®, Cary, NC). Data were filtered (at least 6 obs > background determined with
984 kernel density plots: 28571 probes + standard deviation in all obs. > 0.38), quantile

985 normalized and investigated in a hierarchical clustering and a two-dimensional PCA before
986 differential analysis was performed to visualize the similarity/dissimilarity between
987 individuals.

988 Differential analysis was performed using a general linear model (GLM) that takes the
989 pairing between samples into account for variance and estimate computations. This was
990 followed by moderated t-tests between the differentiation groups (iMACs, prehypertrophic
991 chondrocytes, and hypertrophic chondrocytes). Raw p-values were adjusted for multiple
992 testing with the BH correction to control the FDR.

993 Differentially expressed probes (DEPs) were investigated under a 5% significance
994 threshold (FDR < 0.05) combined with different fold change thresholds ($|FC| > 1, 1.3, 1.5$
995 and 2). Graphical multivariate explorations were performed with the same methodology as
996 previously described.

997 Functional analysis of signatures was performed with Ingenuity® Pathway Analysis
998 (IPA, Qiagen Redwood City, CA, USA). Functional enrichments were identified by
999 Fisher's exact test. The activation status of the functions/pathways was predicted using IPA
1000 by calculating a regulation Z-score and an overlap p-value, which were based on the
1001 number of known genes of interest per pathway/function, the expression changes of these
1002 target genes and their agreement with the literature findings. It was considered
1003 significantly activated (or inhibited) with an overlap p-value ≤ 0.05 and an IPA activation Z-
1004 score ≥ 2.0 (or ≤ -2.0). The detailed descriptions of IPA analysis are available under
1005 “Upstream Regulator Analysis”, “Biological Functions Analysis”, and “Ingenuity
1006 Canonical Pathways Analysis” on the IPA website (<http://www.ingenuity.com>).

1007 **Supplementary Figure 1. Experimental design for cell culture study on murine cells.**

1008 Three sets of experiments for chondrocyte hypertrophic differentiation were designed.
1009 In the first one, 12 independent cultures were performed for microarray analysis and the
1010 characterization of the culture model. Matrix calcification was evaluated on these 12
1011 cultures. mRNAs from 8 among the 12 cultures were used for whole genome
1012 transcriptomic analysis after evaluating the most representative litters of each
1013 differentiation group. The mRNA expression of articular prehypertrophic and hypertrophic
1014 chondrocyte markers was determined on 6 among the 12 cultures, which were chosen at
1015 random. For all the markers studied, the mRNA expression was determined on the 6 same
1016 cultures of chondrocytes. The mRNA expression of IL-34, CSF1R and PTPRZ1 in iMACs,
1017 prehypertrophic and hypertrophic chondrocytes was studied on the same 6 cultures except
1018 one due the depletion of the mRNAs for one culture. It was then replaced by mRNAs from
1019 another culture among of the 12 performed, chosen also at random. The mRNAs of the 6
1020 same culture were also used for the determination of changes in the expression of proteases
1021 and markers of bone formation, osteoclastogenesis and angiogenesis in hypertrophic
1022 relative to prehypertrophic chondrocytes. To the results obtained with these 6 independent
1023 cultures, we added results obtained with cultures coming from the second set of
1024 experiments (6 independent cultures), in which cells were stimulated or not with IL-1 β .
1025 The effect of IL- β was first studied for 3 of these 6 cultures on a panel of genes and then on
1026 a restricted panel of genes for the 3 other cultures. In the third set of experiments, 7
1027 independent cultures were performed to evaluate the response of chondrocytes to IL-34. In
1028 parallel, the effect of IL-34 was studied on 5 independent cultures of osteoblasts.

1029

1030

1031 **Supplementary Figure 2. Cellular model of hypertrophic differentiation**
1032 **characterization using multivariate approaches.**

1033 A) Strategy to select the best litters for microarray analyses. Total variation is computed
1034 (sum for all gene expression and delta calcification values between the hypertrophic and
1035 articular groups) to select the most diverging observations between those extreme
1036 phenotypes. B) PCA using all processed microarray gene expression values revealing the
1037 genes preferentially expressed in each group. C) Double ascendant hierarchical clustering
1038 on all processed microarray gene expression values. Hypertrophy differentiation groups
1039 clustered together naturally based on whole-genome gene expression, suggesting important
1040 signatures. Distance between observations: Euclidean, distance between genes: Pearson
1041 correlation. Aggregation methods: Ward and complete. D) Predicted activation profile
1042 focused on osteoarthritis functions. Z-scores of OA-enriched functions in prehypertrophic
1043 chondrocyte signatures and hypertrophic signatures ($Z\text{-score} > |2|$). Functions with $Z\text{-score}$
1044 < -2 are predicted to be inhibited, whereas functions with $Z\text{-score} > 2$ are predicted to be
1045 activated.

1046

1047 **Supplementary Figure 3. Increased expression of IL-34 and IL-34 receptors with**
1048 **chondrocyte hypertrophic differentiation.**

1049 Human chondrocytes were isolated from the OA cartilage of 7 patients, amplified and
1050 cultured as pellets in a chondrogenic medium and then in a hypertrophic medium. **A-D)**
1051 mRNA expression of Sox 9 (**A**), aggrecan (**B**), Mmp13 (**C**) and Tnap (**D**) was determined
1052 in control chondrocytes cultured in the chondrogenic medium and in hypertrophic
1053 chondrocytes cultured in the chondrogenic medium followed by culture in the hypertrophic

1054 medium. **E)** Pellets of control (panel a) and hypertrophic chondrocytes (panel b) were
1055 analyzed for matrix mineralization by von Kossa staining.

1056

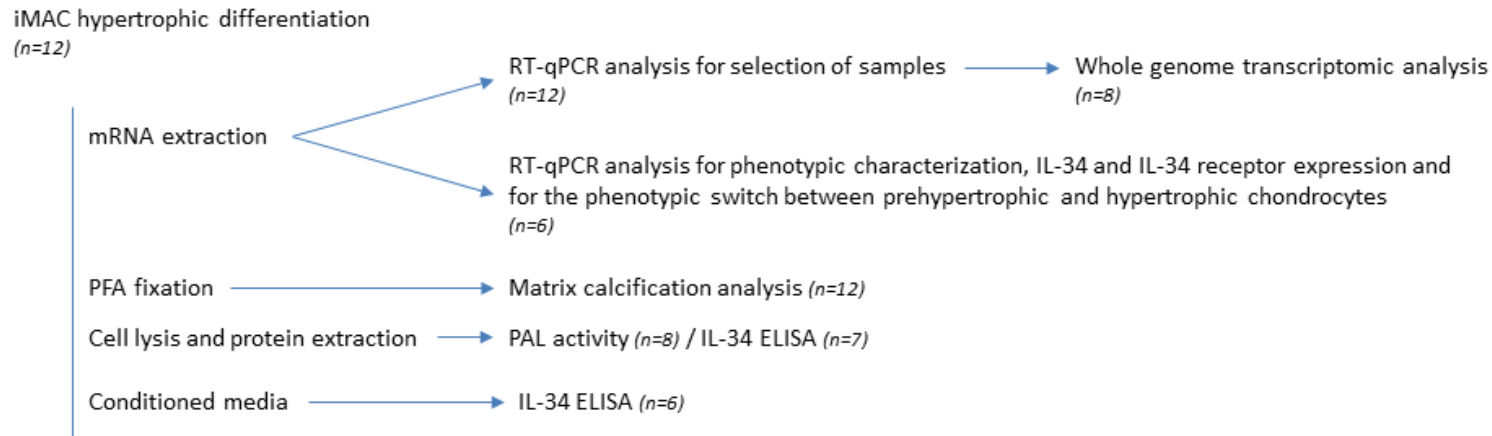
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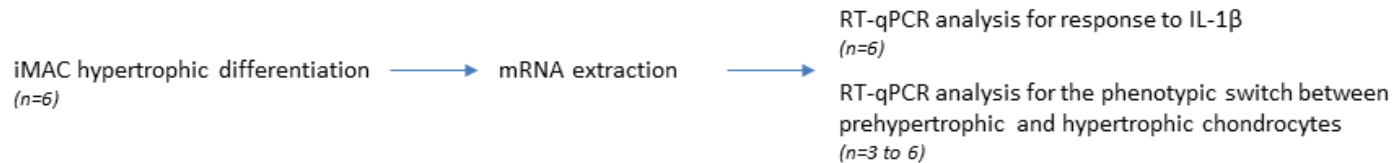
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- 1090
- 1091

Experiment 1. Characterization of iMACs, prehypertrophic and hypertrophic chondrocytes (Figures 1, 2, 3, 4 and S3)



Experiment 2. Effect of IL-1 β (Figure 3)



Experiment 3. Effect of IL-34 (Figures 6 and 7)

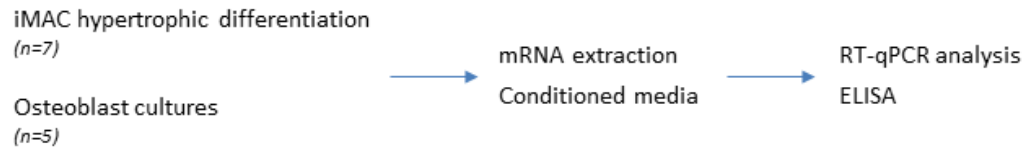
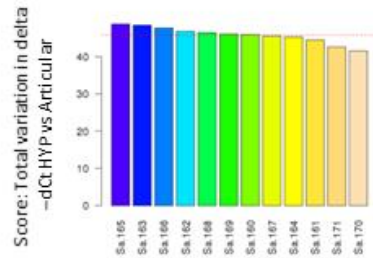
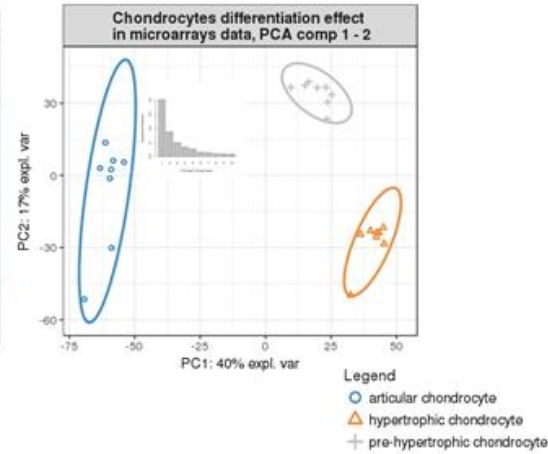
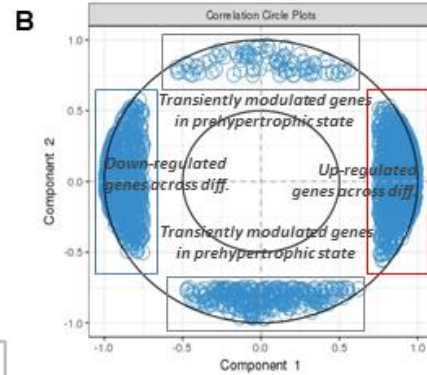
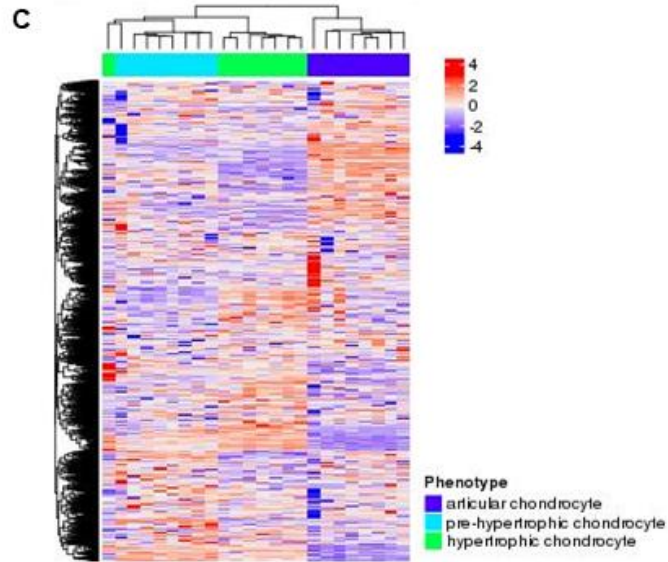


Figure S1

A Selection of most variant litter between Hypertrophic and articular chondrocytes



- $\Delta\Delta Ct_{Hyp-Art}$ cumulating 11 genes from qPCR + calcifications
- Seven litters with a score > mean (scores)
- Final list selection of 8 litters adding the PCA contributions: Sa. 165, 160, 168, 163, 166, 162, 169, 171



D Predicted activation profile focused on osteoarthritis functions

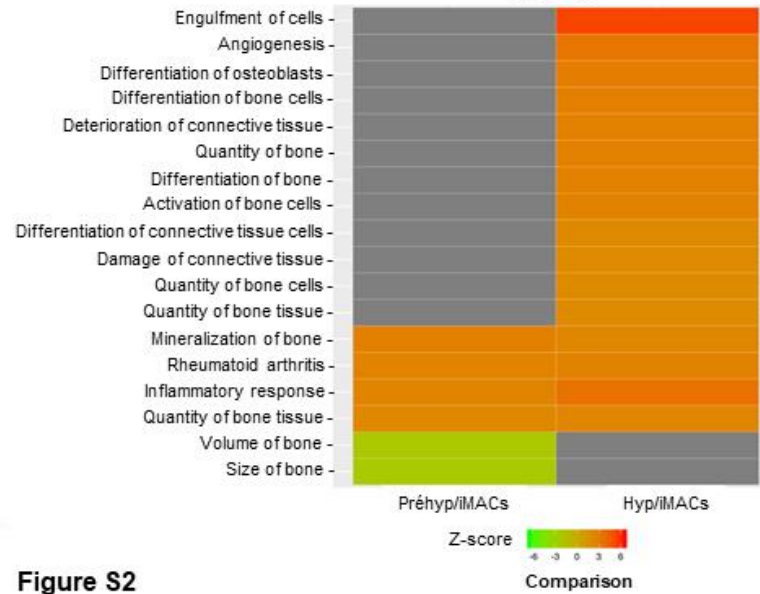


Figure S2

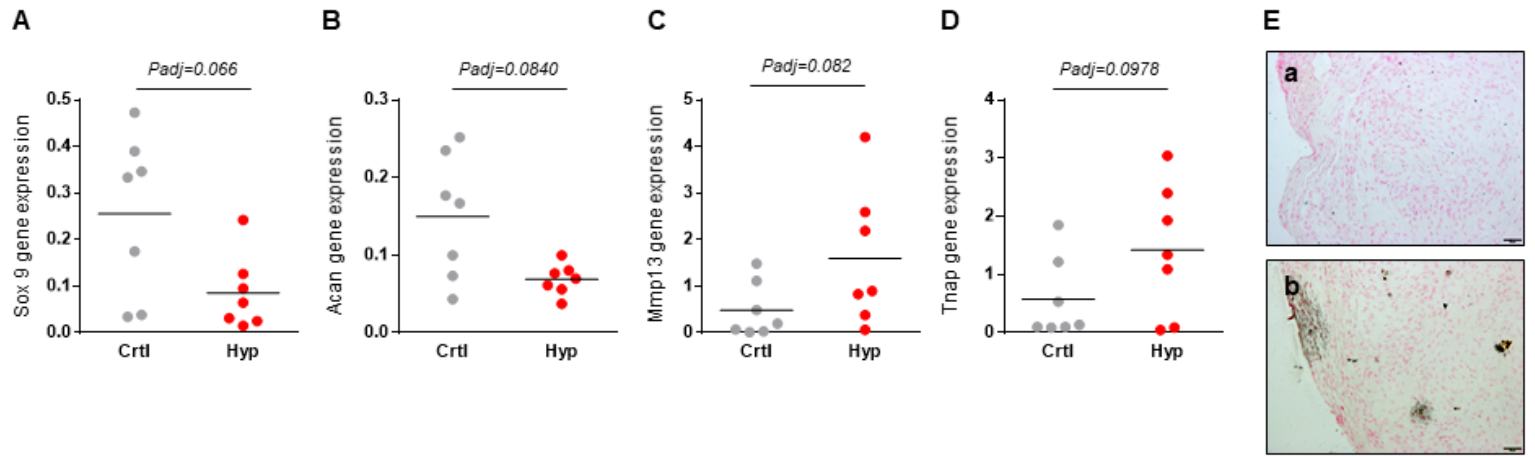


Figure S3

Figure S2. A-B

Osteocalcin Staining linear regression	
Spearman r	0,371
95% confidence interval	0.02184 to 0.6404
P value (two-tailed)	0,033

Osteocalcin (ng/g tissue) linear regression	
Spearman r	0,389
95% confidence interval	0.07454 to 0.6336
P value (two-tailed)	0,014

Figure 1. A-P

RM one way ANOVA (on raw data)

Variable	Pr	
	Mean Diff. (raw values)	95.00% CI of diff.
COL2A1	-0,8519	-0.8898 to -0.8139
ACAN	-0,895	-0.9796 to -0.8104
SOX9	-0,7117	-0.9139 to -0.5094
ChM-I	-0,8727	-0.9087 to -0.8368
Ihh	9,109	2.071 to 16.15
Snorc	0,8967	-0.2019 to 1.995
Osteomodulin	2,098	0.2573 to 3.939
TNAP	36,32	1.283 to 71.35
RunX2	1,559	0.7358 to 2.382
Osterix	5,775	1.083 to 10.47
Col10a1	1,245	-0.4804 to 2.971
MMP13	23,62	-0.3613 to 47.6
Osteocalcin	7,807	1.773 to 13.84
Activity	4,452	0.15 to 8.754
Optical density	0,00725	0.004274 to 0.01023

Figure 3. A-G

RM one way ANOVA (on log transfo)

Variable	IMAC	
	Mean Diff. (log values)	95.00% CI of diff.
Col2a1	-0,2117	-0.4178 to -0.005655
Acan	-0,1274	-0.5346 to 0.2797
IL-6	1,81	1.012 to 2.608
MMP13	1,383	0.6208 to 2.145
VEGF	0,1162	-0.1992 to 0.4316
TSP1	-0,0707	-0.4228 to 0.2814
ChM-I	-0,1587	-0.3658 to 0.0484

Figure 3. H-K

Paired t tests (on raw data)

Variable	Mean of differences vs. Prehyp (raw values)	95.00% CI of diff.
MMP3	21,21	10.29 to 32.14
MMP13	0,4913	-0.9685 to 1.951
ADAMTS4	0,01375	-0.4677 to 0.4952
ADAMTS5	0,12	-0.3716 to 0.6116
TGFB1	0,1033	-0.4510 to 0.6576
BMP2	1,382	0.7836 to 1.980
BMP4	1,246	0.8958 to 1.597
RANKL	21,55	-4.319 to 47.43
CXCL12	3,801	2.192 to 5.41
CCL2	7,73	3.598 to 11.86
OPG	0,7513	0.1318 to 1.371
VEGF	0,9717	0.203 to 1.74
ChM-I	-0,8333	-0.9336 to -0.7331
TSP1	0,535	-0.7466 to 1.817
ANGPTL4	-0,3938	-0.6827 to -0.1048

Figure 4. A-E
RM one way ANOVA (on raw data)

Variable	Pr	
	Mean Diff. (raw values)	95.00% CI of diff.
IL-34	2,955	0.8903 to 5.019
PTPRZ1	33,35	-0.7456 to 67.44
CSF1R	29,57	-19.71 to 78.86
IL-34 supernatant	0,3271	-0.02669 to 0.681
IL-34 Lysate	0,18	0.06573 to 0.2943

Figure 4. F-H
Paired t tests (on log transfo)

Variable	Mean of differences Hyp vs. Ctrl (log values)	95.00% CI of diff.
IL-34	0,6997	0.2273 to 1.172
IL-34 supernatant	1,488	0.8956 to 2.080
IL-34 Lysate	1,722	0.2740 to 3.171

Figure S4. A-D
Paired t tests (on log transfo)

Variable	Mean of differences Hyp vs. Ctrl (log values)	95.00% CI of diff.
SOX9	-0,4762	-0.8016 to -0.1508
ACAN	-0,2857	-0.4608 to -0.1105
MMP13	0,7034	-0.03504 to 1.442
TNAP	0,4004	-0.09972 to 0.9005

Figure 5. A

t-test with Welch correction (on raw data)

Variable	Mean of differences Hyp vs. Ctrl	95.00% CI of diff.
IL-34	264,9	23.43 to 506.4

Figure 6. A-G

RM one way ANOVA (on log transfo)

Variable	Mean Diff. (log values)	95.00% CI of diff.
CXCL12	0,06239	-0.01548 to 0.1403
CCL2	0,1648	-0.1723 to 0.5018
MMP3	-0,03512	-1.217 to 1.147
MMP13	-0,01926	-0.9422 to 0.9036
CCL2 prot	0,2363	-0.003122 to 0.4758
MMP3 prot	0,1776	-0.1418 to 0.497
MMP13 prot	0,03719	-0.1439 to 0.06955

Figure 7. A-I

RM one way ANOVA (on log transfo)

Variable	Mean Diff. (log values)	95.00% CI of diff.
CCL2	0,1863	-0.1877 to 0.5604
MMP3	0,8348	-0.3613 to 2.031
MMP13	0,9762	-0.3911 to 2.343
TNFA	0,6289	-0.6868 to 1.945
PEDF	0,02708	-0.07237 to 0.1265
PTPRZ1	-0,1447	-0.9248 to 0.6355
CCL2	0,1748	0.06643 to 0.2832
MMP3	0,1477	-0.055 to 0.3504
MMP13	0,07507	-0.01046 to 0.1606

ehyp vs. IMACs		
Fold-Changes	Tukey's multiple comparisons test (Adjusted P Value)	Mean Diff. (raw values)
-6,752	0,0001	-0,984
-9,524	0,0001	-0,9915
-3,469	0,0002	-0,84
-7,855	0,0001	-0,9625
10,109	0,0191	14,58
1,897	0,0969	-0,1228
3,098	0,0312	1,038
37,320	0,0441	33,28
2,559	0,0038	2,917
6,775	0,0232	9,678
2,245	0,1382	4,38
24,620	0,0527	23,54
8,807	0,0191	95,28
98,532	0,0435	4,462
1,140	0,0001	0,3642

s vs. Unstimulated		
Fold-Changes (back-transformed)	Dunnett's multiple comparisons test (Adjusted P Value)	Mean Diff. (log values)
-1,628	0,0454	-0,5062
-1,341	0,6357	-0,9513
64,565	0,0016	3,709
24,155	0,0045	1,065
1,307	0,532	0,7516
-1,177	0,8477	-1,269
-1,441	0,1184	-0,4658

Fold-Changes (back-transformed)	P values
22,210	0,0025
1,491	0,4523
1,014	0,948
1,120	0,5579
1,103	0,6786
2,382	0,0019
2,246	0,0001
22,550	0,0852
4,801	0,0008
8,730	0,0048
1,751	0,0241
1,972	0,0178
-5,999	0,0001
1,535	0,3779
-1,650	0,0146

ehyp vs. IMACs		
Fold-Changes	Tukey's multiple comparisons test (Adjusted P Value)	Mean Diff. (raw values)
3,955	0,0127	4,484
34,350	0,054	206,3
30,570	0,219	13,86
7,542	0,0667	0,24
5,320	0,0085	0,255

Fold-Changes (back-transformed)	P values
5,008	0,011
30,761	0,0013
52,723	0,0282

Fold-Changes (back-transformed)	P values
-2,994	0,0116
-1,931	0,0072
5,051	0,0586
2,514	0,0978

Fold-Change	P values
2,813	0,0368

3 vs. 0		
Fold-Changes (back-transformed)	Dunnett's multiple comparisons test (Adjusted P Value)	Mean Diff. (log values)
1,154	0,1091	0,001833
1,462	0,3721	0,1562
-1,084	0,9994	0,2485
-1,045	0,9997	0,2307
1,723	0,0526	0,1928
1,505	0,2886	0,025
1,089	0,6014	0,09637

3 vs. 0		
Fold-Changes (back-transformed)	Dunnett's multiple comparisons test (Adjusted P Value)	Mean Diff. (log values)
1,536	0,2973	0,1056
6,836	0,1399	0,7842
9,467	0,1319	0,804
4,255	0,3211	0,6068
1,064	0,6682	-0,1299
-1,395	0,8431	-0,4129
1,496	0,0098	0,2256
1,405	0,1251	0,1486
1,189	0,0743	0,08721

Hyp vs. IMACs			
95.00% CI of diff.	Fold-Changes	Tukey's multiple comparisons test (Adjusted P Value)	Mean Diff. (raw values)
-0.9861 to -0.9818	-62,500	0,0001	-0,1321
-1 to -0.9827	-117,647	0,0001	-0,09646
-1.003 to -0.6767	-6,250	0,0001	-0,1283
-0.971 to -0.9541	-26,667	0,0001	-0,08982
2.315 to 26.84	15,580	0,0266	5,467
-0.5728 to 0.3272	-1,140	0,6702	-1,02
0.522 to 1.555	2,038	0,0029	-1,06
20.98 to 45.58	34,280	0,0007	-3,039
2.269 to 3.564	3,917	0,0001	1,358
3.395 to 15.96	10,678	0,0094	3,903
0.5042 to 8.257	5,380	0,0322	3,135
-5.246 to 52.32	24,540	0,0964	-0,07917
40.94 to 149.6	96,280	0,0054	87,47
0.3415 to 8.583	98,751	0,0359	0,01019
0.2892 to 0.4391	8,026	0,0001	0,3569

Prehyp vs. Unstimulated			
95.00% CI of diff.	Fold-Changes (back-transformed)	Dunnett's multiple comparisons test (Adjusted P Value)	Mean Diff. (log values)
-0.685 to -0.3275	-3,208	0,0006	-0,7166
-1.624 to -0.2785	-8,939	0,0132	-0,6214
2.576 to 4.843	5116,818	0,0003	2,48
0.01016 to 2.12	11,614	0,0483	0,436
0.2524 to 1.251	5,644	0,0101	0,265
-1.749 to -0.7884	-18,578	0,0008	-0,9472
-0.8436 to -0.08814	-2,923	0,0229	-0,5362

Hyp vs. IMACs			
95.00% CI of diff.	Fold-Changes	Tukey's multiple comparisons test (Adjusted P Value)	Mean Diff. (raw values)
1.513 to 7.454	5,484	0,0102	1,529
85.4 to 327.2	207,300	0,006	172,9
-8.597 to 36.31	14,860	0,2053	-15,72
-0.003997 to 0.484	5,800	0,0532	-0,08714
0.1293 to 0.3807	7,120	0,0028	0,075

30 vs. 0			
95.00% CI of diff.	Fold-Changes (back-transformed)	Dunnett's multiple comparisons test (Adjusted P Value)	Mean Diff. (log values)
-0.1062 to 0.1099	1,004	0,9999	0,0797
-0.2005 to 0.5129	1,433	0,4473	0,2701
-0.5461 to 1.043	1,772	0,6682	0,1745
-0.7613 to 1.223	1,701	0,8153	0,3946
-0.05354 to 0.4391	1,559	0,1174	0,2639
-0.2209 to 0.2709	1,059	0,9775	0,1125
-0.2059 to 0.01311	1,248	0,0797	0,0881

30 vs. 0			
95.00% CI of diff.	Fold-Changes (back-transformed)	Dunnett's multiple comparisons test (Adjusted P Value)	Mean Diff. (log values)
-0.2305 to 0.4417	1,275	0,5837	0,2411
-0.5089 to 2.077	6,084	0,1967	0,6796
-0.7478 to 2.356	6,368	0,2754	0,7012
-0.721 to 1.935	4,044	0,3477	0,7616
-0.2301 to -0.02976	-1,349	0,0213	-0,1457
-0.9801 to 0.1544	-2,588	0,1254	-0,5416
0.07445 to 0.3767	1,681	0,013	0,4146
-0.02479 to 0.3219	1,408	0,0797	0,2832
0.01345 to 0.161	1,222	0,0291	0,1117

Hyp vs. Prehyp		
95.00% CI of diff.	Fold-Changes	Tukey's multiple comparisons test (Adjusted P Value)
-0.1697 to -0.09454	-9,256	0,0002
-0.1868 to -0.006102	-12,353	0,0396
-0.4144 to 0.1577	-1,802	0,383
-0.1302 to -0.04945	-3,395	0,0018
-11.71 to 22.64	1,541	0,5889
-2.136 to 0.09714	-2,162	0,068
-2.778 to 0.6579	-1,520	0,2054
-43.4 to 37.33	-1,089	0,9677
0.5405 to 2.175	1,531	0,0068
-6.132 to 13.94	1,576	0,4704
0.6722 to 5.598	2,396	0,0204
-41.74 to 41.58	-1,003	0,9999
34.33 to 140.6	10,932	0,007
-4.184 to 4.205	1,002	0,9999
0.2838 to 0.43	7,041	0,0001

Hyp vs. Unstimulated		
95.00% CI of diff.	Fold-Changes (back-transformed)	Dunnett's multiple comparisons test (Adjusted P Value)
-1.016 to -0.417	-5,207	0,0013
-1.018 to -0.2247	-4,182	0,0086
1.833 to 3.127	301,995	0,0002
0.08404 to 0.788	2,729	0,0225
0.04916 to 0.4809	1,841	0,0233
-1.324 to -0.5709	-8,855	0,001
-0.7502 to -0.3221	-3,437	0,001

Hyp vs. Prehyp		
95.00% CI of diff.	Fold-Changes	Tukey's multiple comparisons test (Adjusted P Value)
-0.3452 to 3.403	1,223	0,0971
82.31 to 263.6	6,035	0,0037
-54.35 to 22.91	-2,057	0,4429
-0.447 to 0.2727	-1,300	0,7486
-0.1534 to 0.3034	1,338	0,5711

100 vs. 0		
95.00% CI of diff.	Fold-Changes (back-transformed)	Dunnett's multiple comparisons test (Adjusted P Value)
-0.01186 to 0.1713	1,201	0,0828
-0.2201 to 0.7603	1,863	0,2944
-0.7296 to 1.079	1,495	0,8792
-0.4115 to 1.201	2,481	0,3712
-0.06649 to 0.5943	1,836	0,1101
-0.05553 to 0.2806	1,296	0,1846
-0.2153 to 0.03908	1,225	0,1684

100 vs. 0		
95.00% CI of diff.	Fold-Changes (back-transformed)	Dunnett's multiple comparisons test (Adjusted P Value)
-0.09881 to 0.581	1,742	0,1342
-1.051 to 2.411	4,782	0,4424
-1.26 to 2.662	5,026	0,5023
-0.472 to 1.995	5,776	0,1888
-0.2651 to -0.02623	-1,399	0,0262
-1.513 to 0.4296	-3,480	0,2367
0.2119 to 0.6174	2,598	0,0041
0.1544 to 0.4119	1,920	0,0031
0.06202 to 0.1614	1,293	0,0029