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1 **Species variations in tenocytes' response to inflammation require careful selection of animal**
2 **models for tendon research**
3
4 **Author list: Gil Lola Oreff[1+], Michele Fenu[1+], Claus Vogl[2], Iris Ribitsch[1], Florien Jenner[1*]**
5
6 1 University of Veterinary Medicine Vienna, Department of Companion Animals and
7 Horses, Equine Surgery Unit, VETERM, Veterinaerplatz 1, 1210 Vienna, Austria
8 2 University of Veterinary Medicine Vienna, Department of Biomedical Sciences, Institute
9 of Animal Breeding and Genetics, Veterinaerplatz 1, 1210 Vienna, Austria
10
11 **[+] shared first author**
12 *** corresponding author: F. Jenner:** Florien.Jenner@vetmeduni.ac.at
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16
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18 **Abstract**
19 For research on tendon injury, many different animal models are utilized; however, the extent
20 to which these species simulate the clinical condition and disease pathophysiology has not yet
21 been critically evaluated. Considering the importance of inflammation in tendon disease, this
22 study compared the cellular and molecular features of inflammation in tenocytes of humans
23 and four common model species (mouse, rat, sheep, and horse). While mouse and rat
24 tenocytes most closely equalled human tenocytes' low proliferation capacity and the negligible
25 effect of inflammation on proliferation, the wound closure speed of humans was best
26 approximated by rats and horses. The overall gene expression of human tenocytes was most
27 similar to mice under healthy, to horses under transient and to sheep under constant
28 inflammatory conditions. Humans were best matched by mice and horses in their tendon
29 marker and collagen expression, by horses in extracellular matrix remodelling genes, and by
30 rats in inflammatory mediators. As no single animal model perfectly replicates the clinical
31 condition and sufficiently emulates human tenocytes, fit-for-purpose selection of the model
32 species for each specific research question and combination of data from multiple species will
33 be essential to optimize translational predictive validity.
34

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35 **Introduction**

36 Animal models are cornerstones of biomedical and translational medicine research. They are
37 used when it is unethical or impractical to study the target species to explore basic
38 pathophysiological mechanisms, to evaluate safety and efficacy of new treatment approaches,
39 and to decide whether novel therapeutic candidates warrant the economic and moral costs of
40 clinical development.[1-7] For 90% of new treatment strategies, however, translation from basic
41 science to the clinic fails, mainly because clinical trials show them to be inefficient (52%) or
42 unsafe (24%) during phases II and III.[4,5,8] Such translational failures cost animal lives, strain
43 clinical trial volunteers, and burden biomedical research, the pharmaceutical industry and
44 health care systems. So far, attempts to optimize translational success have mainly focused on
45 internal validity flaws such as methodological shortcomings in animal and clinical trials,
46 publication bias, or overoptimistic conclusions about efficacy. Yet another key factor, the
47 external validity, or generalizability, of animal models has received little attention.[4,5,8-13]
48 Common problems of external validity include species differences in disease pathophysiology,
49 common confounding comorbidities and the selection of outcome measures.[5] An animal model
50 should sufficiently emulate aetiology, pathophysiology, symptomatology and response to
51 therapeutic interventions of the target species to allow extrapolation.[5,11] As no single animal
52 model perfectly recapitulates the clinical realm, fit-for-purpose validation and selection of the
53 most appropriate model species is essential.[10-13] Unfortunately, for musculoskeletal disorders,
54 such as tendinopathy, in depth validation studies of animal models beyond structural and
55 biomechanical similarities are largely lacking.

56 Tendinopathy, a disabling overuse injury, is the most common musculoskeletal complaint for
57 which patients seek medical attention.[14] It is prevalent in both occupational and athletic
58 settings, afflicting 25% of the adult population, and accounting for 30–50% of all sport
59 injuries.[15-18] Major tendons experiencing high loads are most commonly affected, especially the
60 weight-bearing and energy-storing Achilles tendon, which routinely experiences loads of up to
61 12.5 times the weight of the individual.[6,19] Many intrinsic and extrinsic factors, including age,
62 body weight and physical loading, influence the aetiopathogenesis of tendinopathy. Overload
63 and repetitive strain lead to accumulation of microdamage and concurrent inflammatory,
64 dysregulated reparative and degenerative processes, causing clinical symptoms, e.g., activity-
65 related pain, focal tendon tenderness and swelling, and functional limitations. Overt clinical
66 symptoms such as pain are preceded by tendinous matrix remodelling, an inflammatory cellular
67 process mediated in part by metalloproteinase enzymes.[20,21] Due to its low cellularity,
68 vascularity and metabolic rate, a tendon's response to injury is inefficient, requiring lengthy
69 periods of recuperation and often resulting in a fibrovascular scar. Scar tissue has significantly
70 inferior biomechanical properties than the original tendon tissue and is prone to re-injury.[16,22-24]
71 Current treatment options are mostly palliative and fail to restore the functional properties of
72 injured tendons.[16,22-24] Tendinopathy thus has a significant adverse impact on quality of life and
73 costs individuals and the society an estimated annual expense of $30 billion.[25,26] This is driving
74 research efforts into unravelling the molecular mechanisms of tendinopathy and developing
75 targeted regenerative therapies. Of particular interest in this context are the cellular and
76 molecular processes orchestrating inflammation in tendinopathy and the mechanisms

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77 governing the development of chronic inflammation that fails to resolve in persistently
78 symptomatic patients.[27]

79 Tendon injury induces a local inflammatory response, characterized by immune cell infiltration
80 and the expression of pro-inflammatory mediators, which in turn reduce collagen production
81 and induce vasodilation, angiogenesis, and matrix metalloproteinase (MMPs) expression.[28-31]
82 Furthermore, the inflammatory milieu can modify tenocyte physiology by increasing metabolic
83 activity and inducing an activated, proinflammatory phenotype with inflammation memory and
84 the capacity for endogenous production of cytokines such as TNF-α, IL-1β, IL-6, IL-10, VEGF and
85 TGF-β.[29,30,32] While the initial inflammatory response is essential to start the healing process,
86 sustained inflammatory conditions contribute to dysregulated matrix remodelling and
87 fibrovascular scarring during healing.[18,31] Chronic inflammation thus drives tendon
88 degeneration before tearing or any other clinical signs of tendinopathy, impairs healing after
89 injury and promotes the development of tendinopathy.[14]

90

91 While human tendon tissue can typically be procured only from individuals with advanced
92 pathology, animal models provide the opportunity to obtain tissue during all stages of
93 tendinopathy to study organ, cellular and molecular changes over the entire course of the
94 disease. In animal models, consistent and repeatable injuries can be induced, evaluated and
95 treated, while controlling for potential confounding influences.[23,33,34] Since no species has been
96 established as the gold standard for tendinopathy research, many induced and spontaneous
97 animal models ranging from small rodents (mice, rats) to large animals (sheep, horses) are
98 utilized.[19,35-43] While the biomechanical properties of the various species are well established,
99 their ability to simulate the pathophysiology of human tendon disease, including the molecular
100 behaviour of key genes and pathways, has not been critically evaluated yet and detailed
101 analyses of species- specific differences in cytokine expression and regulation as well as of
102 tenocytes susceptibility to cytokines are still lacking.[44]
103 Considering the importance of inflammation in tendon disease,[29,33,45,46] this study compares the
104 cellular and molecular features of inflammation in tenocytes of humans and four common
105 model species (mouse, rat, sheep, and horse) to aid in the evidence-based selection of fit-for-
106 purpose translational animal models for tendon research. Mice and rats are included due to
107 their prevalent use as laboratory animals and availability of species-specific molecular tools.[6,47]
108 Larger animals are used increasingly as translational models due to their more comparable
109 tendon dimensions and biomechanics.[23,48-50] Horses present an attractive model of human
110 tendinopathy since their superficial digital flexor tendon is a weight-bearing and energy-storing
111 tendon analogous to the human Achilles tendon, which is similarly prone to naturally occuring
112 tendon disease with high recurrence rates.[9,51,52] Furthermore equine ageing proceeds similarly
113 to humans.[39,51,52] Sheep are included because features of clinical tendinopathy of horses could
114 be emulated also in ovine induced models.[51,53-57] In particular, the ovine intra-synovial tendon
115 lesion model mimics the clinical intra-synovial tendon disease of humans and horses more
116 accurately than small animal extra-synovial models, e.g., with respect to histology and gene
117 expression, to similarities in the biomechanical environment and to failure of lesions to
118 heal.[51,53-57]

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119

120 **Results**

121 Morphology

122 Tenocytes from all five species shared common characteristics with a fusiform appearance,
123 adherence to the flask and similar dimensions (fig. 1): human tenocytes measured 177.8 ± 40.1
124 µm (mean ± s.d.) in length and 20.2 ± 4.4 µm in width, mouse 163.7 µm (±23.6) x 19.4 µm
125 (±3.3), rat 182.5 (±26.6) x 24.2 (±8.0), sheep 206.3 (±45.5) x 28.9 (±8.5) and horse 193.2 (±8.4) x
126 15.4 (±1.4). In high confluency, tenocytes from human, sheep and horse showed similar
127 morphology, creating cell bundles arranged in a storiform pattern (fig. 1A), while tenocytes
128 from mouse and rat had a more scattered appearance with a random orientation.
129

130 Proliferation assay

131 Proliferation, migration and gene expression of tenocytes of all five species were compared
132 under standard culture conditions (healthy control) as well as under transient (24 h) and
133 constant exposure to inflammatory stimuli (fig. 2). Under healthy conditions (fig. 3, table 1),
134 equine tenocytes had the highest proliferation rates while murine cells had the lowest. Human
135 tenocytes exhibited the second lowest proliferation capacity with an inability to double the cell
136 amount over the 48h observation period. Sheep and rat tenocytes were in the middle. From fig.
137 3, it can be seen that slopes are quite variable among species, but within species variation is
138 low. Therefore, the slopes of tendon cells of all four model species are significantly different
139 from those of humans ($p<0.001$; to correct for multiple testing using Bonferroni with four
140 comparisons, the nominal significance levels, 0.05, 0.01, and 0.001, are set to the corrected
141 levels: 0.0125, 0.002, 0.0002, respectively), even for only three biological replicates per species.
142 Under constant exposure to inflammatory stimulation (10 ng/ml IL1β and 10 ng/ml TNFα), the
143 proliferation of sheep tenocytes decreased significantly and fell to about human levels, i.e., the
144 difference in the proliferation slopes between healthy and constantly inflamed sheep
145 decreased significantly ($p<0.01$). All other differences in slopes between healthy, constantly and
146 transiently (only 24h inflammatory stimulation) inflamed conditions were not significant.
147

148 Wound healing (scratch) assay

149 Gap closure was significantly different (in all cases $p<0.001$) between all conditions, fastest for
150 healthy and slowest for constantly inflamed cells of all species (fig. 3, table 1). Murine tenocytes
151 showed the slowest wound healing for all conditions. There was no statistically significant
152 difference in migration speed between healthy and transient inflammatory conditions in
153 tenocytes of any species and between healthy and continuously inflamed conditions only for
154 mouse and rat cells ($p< 0.05$).
155 Under healthy conditions, gap closure of tenocytes from all species except rats was significantly
156 different (in all cases $p< 0.003$) from humans with cells from rats showing the fastest wound
157 healing (gap closure at 48h mean 90.08% ± 8.01% s.d.), closely followed by humans (gap closure
158 at 48h mean 86.25% ± 2.47% s.d.) and cells from mice the slowest (gap closure at 48h mean
159 51.49% ± 10.23% s.d.). Under transient inflammation rat tenocytes again were fastest (gap
160 closure at 48h mean 64.81% ± 7.84% s.d.), with ovine (gap closure at 48h mean 58% ± 11.17%
161 s.d.), human (gap closure at 48h mean 56.06% ± 25.27% s.d.) and equine tenocytes (gap closure
162 at 48h mean 55.71% ± 9.6% s.d.) following with similar wound healing rates, while murine

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163 tendon cells again were slowest (gap closure at 48h mean 29.46% ± 4.36% s.d.). Under constant
164 inflammation, equine tenocytes were fastest (gap closure at 48h mean 35.23% ± 8.98% s.d.)
165 followed closely by human tendon cells (gap closure at 48h mean 34.99% ± 19.12% s.d.). The
166 change in migration speed compared to healthy was significantly different from human
167 tenocytes for ovine tendon cells under transient and equine and murine tenocytes under
168 constant inflammation (table 1).
169
170 Quantitative PCR
171 The species show variable approximations of human expression levels among functional gene
172 groups and conditions (table 2 and 3, fig. 4 and 5, suppl. fig. 1 and 2, suppl. table 1).[58] A
173 univariate Analysis of Variance (ANOVA) demonstrated significant differences between each
174 species and humans in many genes relevant for tendon function and inflammatory response
175 (table 2). Remarkably, healthy tenocytes of all four species show significant differences to
176 humans in their expression of Col1, the main tendon matrix component, of collagenase MMP13
177 and of the key inflammatory mediator COX2. Similarly, significant differences from humans in
178 the COX2 expression of transiently inflamed tenocytes are evident for all four species and in IL6
179 expression for all species except rats. In contrast, no significant differences to humans were
180 seen in MMP1 expression for any species and the osteogenic marker ALP was only significantly
181 different in healthy murine tenocytes. NFkB expression exhibited a significant difference only in
182 healthy horse tendon cells and p53 in healthy horse and rat tenocytes.
183 To condense the information from the univariate ANOVA results to overall measures of
184 similarity between the different animals and humans we calculated the multivariate
185 Mahalanobis distances of the four species to humans. The Mahalanobis distance is a non-
186 dimensional measure of dissimilarity, where between group distances are weighted by the
187 inverses of within group variability, much like the test statistic of a t-test for one variable.
188 Similar to its use in graphically detecting outliers in multiple dimensions, we use it to show
189 multivariate dissimilarity in gene expression among species. In supplementary figure 1, we
190 summarize conditions within a gene. From figure 4, it is evident that Cox2 is rather variable
191 among species (especially human healthy cells differ from all animals, least so from mice) while
192 showing relatively little variation within species under all conditions. Hence the Mahalanobis
193 distances to humans are comparatively large with mice being closest to humans (suppl. fig. 1).
194 The second immune gene, Il6, shows also relatively little within species variation (fig. 4), but
195 comparatively less between species variation; especially rats and sheep are similar to humans,
196 while mice and horses are slightly further away (fig. 4, suppl. fig. 1). The Mahalanobis distances
197 (table 3) of the immune genes are relatively large compared to the other gene combinations
198 due to low within species variability. The overall distance of the immune genes is a compromise
199 between the two genes, such that rats are slightly more similar to humans than mice, with
200 sheep and horses further away. When different functional groups of genes are analysed,
201 variable Mahalanobis distances of the four model species from humans are found (table 3).
202 While mice and horses are closest to humans in their tendon marker and collagen expression,
203 horses appear to be by far the best model for MMPs (table 3). For the overall gene expression
204 of healthy tenocytes, Mahalanobis distances from human tenocytes are large and similar
205 among species, with mouse tendon cells appearing least and rat tenocytes most distant from
206 human. For transient inflammation, Mahalanobis distances to humans are generally lower, but

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207 the spread of differences widens, with horses, sheep and mice relatively close and rats clearly
208 furthest. For constant inflammation, the pattern is qualitatively similar to that under transient
209 inflammation. Overall, the pattern of multivariate dissimilarity of species varies widely and
210 unpredictably among species pairs with no single species most similar to humans.
211
212
213 Principal Component Analysis (PCA) is an exploratory technique for reducing the complexity of
214 data. We used expression data from all genes and plot the results for the different species x
215 condition combinations (fig. 5, suppl. fig. 2). The plot of PC1 vs. PC3 is easier to interpret than of
216 other combinations of PCs. (fig 5). With the exception of humans and mice, species are
217 generally not well-separated. Within all species, the different conditions are spread along an
218 oblique line with healthy to the upper left and inflamed to the lower right. Within mice, healthy
219 is also separated from inflamed, while in sheep the pattern is less clear. Pairwise plots of other
220 PCs show similar but less clear patterns (suppl. fig. 2).
221
222 **Discussion**
223 Choice of the most appropriate animal model is the most essential and challenging element of
224 animal-based research, and also an important aspect of the 3Rs (i.e., replace, reduce, refine) to
225 ensure the best use of animals.[59-61] Unfortunately, the choice of animal models frequently is
226 based more on convention, financial and practical considerations, such as housing and
227 husbandry requirements or the availability of reagents and biochemical tests, than compelling
228 scientific evidence of the fit to human diseases and clinical contexts.[4,5,9,62-64] The lack of formal
229 requirements for animal models is due to the traditional assumption that genetic homology
230 derived from a common evolutionary origin also implies functional similarities of gene
231 regulation, signalling pathways and developmental systems between species (the "unity in
232 diversity" concept).[7] Species may, however, differ in critical aspects and rarely have assumed
233 similarities been empirically demonstrated.[7] The diversity of human patients and symptoms is
234 thus unlikely fully represented in highly inbred rodents.[65,66] Even humanized models, which
235 have contributed significantly to research by facilitating functional studies in vivo, cannot
236 replicate the complexity of human disease.[67]
237
238 Both the European Medicines Agency (EMA) and the USA Federal Food and Drug Administration
239 require the use of fit-for-purpose animal models to evaluate efficacy, durability, dose-response,
240 degradation and safety of new therapeutics for market approval. Recently, these regulatory
241 authorities published guidelines identifying requirements to demonstrate the relevance of
242 animal models for investigational new product testing by cross-species comparison of the
243 structural homology of the target, its distribution, signal transduction pathways and
244 pharmacodynamics.[68,69] Furthermore, several voluntary initiatives have established criteria to
245 encourage the evidence-based selection of animal models for stroke and schizophrenia.[11,70,71]
246 To this end, both the model species and disease-induction protocols, need to be validated by
247 comparing the animal model with the gold standard or the target species.[11] As no gold standard
248 for tendon research is available, this study compared tenocyte morphology, proliferation rate,
249 wound healing speed, and gene expression of two small animals (mouse and rat) and two large
250 animals (sheep and horse) to human under healthy as well as "diseased" (transiently and

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251 continuously inflamed) conditions to determine similarities and differences among species. It
252 can serve as the foundation for a rational, evidence-based choice of optimal animal models for
253 specific aspects of human tendinopathy.
254
255 Tendon injury induces a local inflammatory response, which initiates the healing process.
256 Tendon healing occurs in three chronologic phases: inflammation (0-7 days), proliferation (1-6
257 weeks), and remodeling (6 weeks – 6 months). While these stages overlap, they are
258 characterized by temporally and functionally distinct cytokine profiles and cellular processes.[72]
259 The initial inflammatory phase is characterized by influx of inflammatory cells, which release
260 chemotactic and proinflammatory cytokines and growth factors that lead to recruitment and
261 proliferation of macrophages and resident tendon fibroblasts.[44,72-83] In addition, tenocytes
262 produce also several endogenous cytokines and growth factors which contribute to the healing
263 process in an auto- and paracrine manner.[44,75] During the proliferative stage tenocytes
264 proliferate and produce an immature neomatrix with a predominance of type III rather than
265 type I collagen.[44,72,80-82,84] Lastly, in the course of the remodeling phase, the cellularity
266 decreases, matrix synthesis is reduced and collagen fibrils and tenocytes align linearly with the
267 direction of tension.[44,72-79,81-83] However, in both man and horse suffering from naturally
268 occurring tendon disease, the normal architecture, composition and function of the tendon are
269 never completely restored, predisposing them to recurring injury and tendinopathy.[44,72-79,81-83]
270
271
272 Given the importance of inflammation in tendon injury and repair, with pro-inflammatory
273 cytokines acting as a regulatory link between several catabolic and anabolic systems and as a
274 double-edged sword both promoting and impeding tendon repair,[44,83,85-88] this study focused on
275 the comparative response to inflammation.
276 We used IL-1β and TNF-α, two hallmark cytokines of inflammation in tendons, which are
277 associated with tendon injury and tendinopathy in vivo and in vitro, to induce disease-relevant
278 inflammation.[28,30,33,45,89-97] IL-1 activates the NF-κB pathway in tenocytes, induces the
279 production of inflammatory mediators including COX2 and IL6, and matrix remodelling factors
280 such as MMP1, MMP3 and MMP13.[28,30,89-91] It can even cause loss of the tenocyte phenotype,
281 which is associated with decreased expression of tendon-related genes, e.g. COL1, SCX and
282 TNMD.[28,30,89-91] Similarly TNF-α can strongly activate tenocytes, stimulating them to produce
283 more cytokines, including IL-1β, TNF-α, IL- 6.[28,30,92-94,96] Accordingly, we used tendon-specific
284 markers (SCX, TNC, TNMD, COL1, COL3, COL5), matrix remodelling proteinases (MMP1, MMP3,
285 MMP13) and inflammatory factors (COX2, IL6, NF-κB, p53) in addition to proliferation and
286 wound healing speed as read-outs to evaluate the response to IL-1β/ TNF-α induced
287 inflammation.
288
289 Interestingly, while mouse and rat tenocytes most closely matched human tenocytes' low
290 proliferation capacity and minimal effect of inflammation on proliferation, the human wound
291 closure speed was best approximated by rats and horses. Tenocyte migration to the injured
292 tissue and proliferation are essential processes in tendon healing.[98,99] Accordingly,
293 inflammatory stimulation, e.g. with IL-1β, has been shown to increase tenocyte migration and
294 proliferation, the capacity for which decreases with age.[80,83,87,88,100-106] In this study, we

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295 observed a decrease in tenocyte migration and proliferation following inflammatory stimulation
296 in all species (statistically significant for sheep tenocyte proliferation as well as rat and mouse
297 tendon cell migration under constant inflammation) except rats (non-significant trend toward
298 increased proliferation), which may be due to our use of tenocytes from individuals in disease-
299 relevant age groups.
300
301 The overall gene expression of human tenocytes was most similar to murine under healthy,
302 equine under transient and ovine under constant inflammatory conditions. The species
303 difference between human and the four animal models was particularly evident in the
304 expression of the main tendon matrix component COL1. Healthy tenocytes of all four model
305 species exhibited significant differences to human in their expression of COL1. Col1 typically
306 amounts to appr. 95% of total tendon collagen or 50-80% of tendon dry weight,[107] but
307 cytokines, such as IL-1β and TNF-α, suppress COL1 synthesis, which leads to reduced
308 stiffness.[28,44,84,108] In this study the decrease in COL1 synthesis following inflammatory
309 stimulation could be observed in all species and was most pronounced in rat and mice, least in
310 sheep and most similar to humans in horses.
311 The expression of the transcription factor SCX, a specific marker of the tendon/ligament
312 lineage,[109] while low in all species under healthy conditions, only increased in humans upon
313 constant inflammation. SCX is a transcription factor that regulates tendon genes, including Col1
314 and Tnmd, and is required for normal tendon development[110,111] and adult tendon repair in
315 mice.[112,113] An increase in its expression is likely to result in changes in the expression of its
316 downstream genes and to be beneficial to tendon healing post injury.[113] The essential
317 contribution of SCX was also shown in SCX-null mice, which fail to convert from producing
318 primarily COL3 to synthesizing mainly COL1 during tendon repair, supporting the hypothesis
319 that the transcriptional control of collagen type I is mediated by SCX.[113] Overall for the six
320 tenogenic factors, rat tenocytes showed the largest difference to humans in the Mahalanobis
321 distance, while tendon cells from mice and horses most closely equaled humans, indicating that
322 these species might be most suitable for studies evaluating ECM production and tendon
323 healing.
324 For matrix remodelling proteinases, the species differences were most prevalent for healthy
325 tenocytes: tendon cells of all model species differed from humans for MMP13 and all but
326 horses for MMP3. MMPs are key players in physiological and pathological tendon ECM
327 remodeling, contributing to the degradation of tendon ECM and hence the loss of the
328 biomechanical resistance and durability of tendon.[44,114-116] An increase in MMP expression has
329 also been implicated in the pathogenesis of tendinopathy.[44,116] MMP13 specifically was
330 upregulated in rotator cuff tendon tears and flexor tendon injury.[117-119] In this study,
331 inflammatory stimulation increased MMP13 expression in tenocytes of all species, only
332 minimally in mice and horses but 4-8-fold in rats, sheep and humans. In contrast, all species
333 showed similarly increased MMP1 expression following inflammatory stimulation; no significant
334 differences were observed in MMP1 expression in any species in any condition compared to
335 humans. This corresponds well with other studies showing upregulation of MMP1 in ruptured
336 tendons suggesting a high level of collagen degradation by this enzyme.[120] In total, for the
337 functional group of ECM remodelling genes, horses again provided the best and rats the worst
338 match to humans as shown in the Mahalanobis distance analysis.

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339 For the expression of inflammatory mediators, the Mahalanobis distances of all species were
340 larger than for the other functional gene groups. Although the immunophysiology of larger
341 animal species has traditionally been presumed to be closer to humans than rodents,[47,121] rat
342 tenocytes most closely approximated human tendon cells in this category. Additionally, in
343 healthy condition, mice presented the lowest distance from all animals, rising again the
344 question if larger animals truly are more similar to human. Remarkably, healthy and transiently
345 inflamed tenocytes of all four model species, as well as constantly inflamed ovine and equine
346 tenocytes, showed significant differences to human in their expression of COX2. Following
347 inflammatory stimulation, COX2 was only significantly upregulated in humans, mice and rats.
348 Upregulation of COX2 plays an important, multifaceted role in the inflammatory cascade in
349 injured tendons through the synthesis of prostaglandins.[125] COX2 is essential in the early injury
350 response as evidenced by impaired tendon repair following administration of selective COX2
351 inhibitors in the early repair phase.[122] The lacking upregulation of sheep and horses therefore
352 invites further investigation into the early tendon healing response in the different species *in*
353 *vivo*.
354 Correspondingly, IL6, a cytokine with strong association with inflammation in tendon
355 disease,[58,123,124] displayed significantly different expression in transiently inflamed tenocytes of
356 all species except rats. Statistically significant differences in IL6 expression compared to human
357 were also evident under constant inflammatory conditions for mice and horses. IL6 plays an
358 essential role in tendon healing as repair processes in IL6 knock-out mice are impaired.[38] It
359 tenocytes in two ways: i) IL6 stimulates tenocyte proliferation and survival and ii) it inhibits
360 their tenogenic differentiation via the Janus tyrosine kinases/Stat3 signaling pathway.[44,125]
361
362 Cell properties may be influenced not only by species and interdonor differences but also by
363 cell isolation and processing methods.[126,127] In the present study, two isolation methods,
364 enzymatic digestion and cell migration out of tendon explants, have been used depending on
365 the available sample size. Enzymatic digestion was used for smaller sample sizes as higher cell
366 yields are achieved with this method, while the explant technique is less invasive and requires
367 less manipulation and labour. As both methods were used for all species and alterations in
368 experimental conditions have been shown to be of minor importance to cell behaviour
369 compared to cell source and interdonor variability,[126] the isolation method is unlikely to have
370 significantly influenced the species-specific gene expression profiles observed in this study.
371
372 In summary, the results of our study show that all four model species approximate some
373 aspects of the behaviour of human tenocytes well and others poorly. No animal model
374 sufficiently emulates human tenocytes' cellular and molecular features and response to
375 inflammation to be considered the gold-standard for tendon research. Translational medicine
376 will need to continue to rely on a fit-for-purpose selection of animal models to approximate the
377 human condition, based on the essential characteristics that must be mimicked for a particular
378 research question.[19] Peculiarities, strengths, and weaknesses of the model species need to be
379 accounted for in the study design, analysis and interpretation.[19,128,129] Data from multiple
380 animal models should be combined to optimize translational predictive validity.
381
382 **Materials and Methods**

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383 Tenocytes of four mammalian species (mouse, rat, sheep, horse) were compared with human
384 tenocytes (n = 3 donors, i.e., biological replicates, per species). All methods and experimental
385 protocols in this study were carried out in accordance and compliance with relevant
386 institutional and national guidelines and regulations.
387
388 Tenocyte isolation from animals
389 All animals were euthanized for reasons unrelated to this study. Based on the "Good Scientific
390 Practice. Ethics in Science und Research" regulation implemented at the University of
391 Veterinary Medicine Vienna, the Institutional Ethics Committee ("Ethics and Animal Welfare
392 Committee") of the University of Veterinary Medicine Vienna does not require approval of in
393 vitro cell culture studies, if the cells were isolated from tissue, which was obtained either solely
394 for diagnostic or therapeutic purposes or in the course of institutionally and nationally
395 approved experiments.
396 Species-specific, energy-storing, weight-bearing tendons were harvested from skeletally mature
397 animals immediately following euthanasia: Achilles tendons from sheep (Merino-cross breed,
398 female, aged 2-5 years), rats (Fischer344 breed, female, aged 3-4 months) and mice (c57bl/6
399 breed, female, aged 8-12 weeks); superficial digital flexor tendons from the front limb of horses
400 (7-15 years, geldings). Under sterile conditions, the paratenon was removed and the tendons
401 were sectioned into small pieces ($<0.5 \times 0.5 \times 0.5$ cm). Isolation of cells was performed either by
402 enzymatic digestion using 3 mg/ml collagenase type II (Gibco Life technologies, Vienna, Austria)
403 for 6-8 hours or migration from explants (explants were removed after 7-10 days) or a
404 combination of both. Cells were expanded until 80 - 90% confluency before passaging.
405
406 Human tenocytes
407 Human tenocytes obtained with ethical approval and informed consent from the Achilles
408 tendon of three male human donors (aged 60-90 years) in accordance with relevant guidelines
409 and regulations (Declaration of Helsinki) were purchased in cryopreserved condition in passage
410 two from two different providers (Pelo Biotech GmbH, Germany and Zen-Bio, North Carolina,
411 USA with review of the protocols and consent forms by an independent review board
412 (Institutional Review Board, Pearl Pathways, LLC) which is accredited by the Association for the
413 Accreditation of Human Research Protection Program Inc.).
414
415 Cell culture
416 The culture medium was identical for all species: minimal essential medium (α-MEM, Sigma-
417 Aldrich, Vienna, Austria) supplemented with 10% fetal bovine serum (FBS-12A, Capricon,
418 Ebsdorfergrund, Germany), 1% L-Glutamine (L-Alanyl L-Glutamine 200Mm, Biochrom), 100
419 units mL^{-1}penicillin and 0.1mg mL^{-1}streptomycin (P/S, Sigma-Aldrich, Vienna, Austria).
420 Cells were cultured at $37^{0}C$, 5% CO2 until the desired passage and number of cells was
421 obtained. Experiments were performed with cells either in passage 3 or 4.
422
423 Morphology
424 Cells were imaged both at low and high confluency using the EVOS FL Auto imaging system in
425 phase contrast with a 40x and 400x objective (ThermoFisher Scientific, AMEP4680). Cell

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426 phenotypes and cell sheet patterns were characterised for all species and compared to human
427 cells. Tenocyte dimensions (length and width) were measured for each of the five species.
428
429 Inflammatory stimulation
430 Gene expression, proliferation and migration of tenocytes of all five species were compared
431 under standard culture conditions (healthy control) as well as under transient (24 h) and
432 constant exposure to inflammatory stimuli (10 ng/ml IL1β (Immuno Tools, Friesoythe,
433 Germany) and 10 ng/ml TNFα (Immuno Tools, Friesoythe, Germany)).[Dakin:2018bi 96] After 24-hour
434 exposure to inflammation, the transient inflammation group received fresh culture medium,
435 while for the constant inflammation group fresh medium was again supplemented with
436 inflammatory factors (fig. 2).
437
438 Proliferation assay
439 Tenocytes were plated in 96-well plates (3000 cells/well in technical triplicates) and cultured
440 under control (healthy), transient and constant inflammatory conditions. The cell number per
441 plate was quantified via DNA fluorescence using the CyQuant assay (Invitrogen) according to
442 the manufacturer's recommendations on day 0, 1, 2, and 3 (fig. 2). As cell proliferation sets in
443 after a lag time of about 24 h and relative proliferation rates decrease steadily, we used log(cell
444 nr) as the target variable and log(time in hours minus 23) as regression variable for the
445 parametric statistical analysis.
446
447 Wound healing (scratch) assay
448 Migration of tenocytes was evaluated in a wound healing model using a magnetic scratch
449 device to create standardized cell-free gaps of 1.5 mm width in confluent sheets of
450 tenocytes.[130] Cells were seeded in 12-well plates (100,000 cells/well in technical triplicates) and
451 left to adhere overnight. Inflammatory stimuli were added to the transient and constant
452 inflammation groups and scratches were created 48 hours after seeding under control
453 (healthy), transient and constant inflammatory conditions (fig. 2). The cell-free area was imaged
454 at 24h intervals (0, 24, 48, 72, 96 hours, fig. 2) in phase contrast using the EVOS FL Auto imaging
455 system with a 4x fluorite objective using coordinate recovery function. The gap size was
456 measured using the MRI Wound healing Tool (http://dev.mri.cnrs.fr/projects/imagej-
457 macros/wiki/Wound_Healing_Tool) in ImageJ (https://imagej.nih.gov/ij/, version 2.0.0-rc-
458 43/1.50e). As gap closure approached 100% in the fastest group, healthy rat tenocytes, at 48h,
459 this time point was chosen as cut-off for slope calculations and comparison of conditions. For
460 the parametric analysis, we used the untransformed gap area [mm^2] as target variable and the
461 untransformed time between 0 hours and 48 hours (before the gap closed in any of the
462 samples) as regression variable.
463
464 Quantitative PCR
465 Tenocytes were seeded in 12-well plates (100,000 cells/well in technical triplicates) and
466 cultured under control (healthy), transient and constant inflammatory conditions. Cells
467 were harvested for RNA isolation using RNA isolation reagent (Trizol, ThermoFisher Scientific,
468 MA, USA) 48 hours after initiation of inflammation, as previously described.[131] The 48 hours

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469 time point was chosen as it allows assessment of the response to inflammation as well as to
470 removal of inflammatory stimuli.
471 Briefly, a solution of Trizol and Chloroform (Sigma-Aldrich) in a ratio of 5 to 1 was used. Total
472 RNA was recovered by the addition of isopropyl alcohol (Sigma-Aldrich) and glycerol (Thermo
473 Scientific). The mixture was incubated on ice and centrifuged for 45 minutes at 13,000 rpm. The
474 total RNA pellet was washed with 75% ethanol and solubilized in RNase-free water. Genomic
475 DNA was removed by a DNA removal kit (Life Technologies, Carlsbad, California, USA). Two
476 nanograms of RNA from each sample was used for the qPCR reaction (qPCR One-Step Eva
477 Green kit, Bio&Sell, Feucht, Germany).
478 We measured gene expression of tendon markers (TNC, TNDM, SCX), collagens (COL1, COL3,
479 COL5), matrix-metalloproteinases (MMP1, MMP3, MMP13), inflammatory factors (IL6, COX2,
480 NFkB, p53), a marker for aberrant tenocyte differentiation (ALP) and for focal adhesion and
481 migration (FAK) in the four model species and humans under healthy, transiently and constantly
482 inflamed conditions. All primers were designed using the Primer3 software. Primer sequences
483 are shown in supplementary table 2. The transcript level for the 15 genes of interest was
484 normalized to the transcript level of the housekeeping gene glyceraldehyde-3-phosphate
485 dehydrogenase (GAPDH) and presented as ratio to GAPDH.[58] The ratio between COL 1 and COL
486 3 was also evaluated for further matrix remodelling characterization, with a higher COL1:COL3
487 ratio indicating a stronger tenogenic phenotype.[132,133] For the parametric analysis, we used the
488 $\log_2$ transformed ratios of the target gene to GAPDH as target variable.
489
490 <u>Statistical analysis</u>
491 For statistical analyses, the R statistical programming language[134] and GraphPad (version 8.4.2)
492 were used. Target and regression variables (where appropriate) are given in the respective
493 subsections. Data are presented descriptively as mean and standard deviation. Generally, linear
494 models (analyses of variance and covariances, ANCOVA) were used, e.g., for wound healing the
495 untransformed area was the target variable, *time* a regression variable, and *species* and
496 *condition* factors; as interactions of two explanatory variables *time*species*, *time*condition*,
497 *condition*species*, and *biological replicate* nested within *species* were included; furthermore,
498 the three-way interaction *time*species*condition* was also included. Note that all terms with
499 *time* are to be interpreted as slopes or differences in slopes. The Tukey's HSD (honestly
500 significant difference) test was used to account for multiple testing, where appropriate.
501 Confidence intervals of parameter estimates were calculated.
502
503 Note that many different target variables are available, i.e., data are multidimensional. With
504 qPCR alone, 15 genes of interest were measured under three conditions. For each gene
505 separately, an ANOVA with species and condition was calculated. Furthermore, we condensed
506 information by calculating the multivariate (Mahalanobis) distance of the $\log_2$-transformed
507 mRNA concentrations, for the three conditions of each gene, and report the distance of each of
508 the four mammalian species from the human values. For a single condition, all 15 genes of
509 interest could be used for calculating the multivariate distance. Additionally, we grouped genes
510 into classes, e.g., all collagens or all matrix-metalloproteinases and calculated multivariate
511 distances for the classes separately, this time jointly for the different conditions. We also
512 calculated a principal component analysis (PCA) of the $\log_2$-transformed qPCR data for all gene,

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513 treatment, and species combinations together. The proportions of the variance explained by
514 the different PC's are reported and the rotated data for the different treatment and species
515 combinations are shown in graphs for the most important components.
516

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852
853 **Conflict of Interest:**
854 The authors have no competing interests to declare.

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855 **Figure Legends**
856
857 Figure 1: Micrographs of tenocytes derived from the Achilles tendon in the mouse, rat, sheep
858 and human or the superficial digital flexor tendon in the horse. Figure 1A shows the tenocytes
859 at a magnification of 40x (scale bar: 1000µm), while figure 1B was taken at a 400x magnification
860 (scale bar: 100µm).
861
862
863 Figure 2: Study timeline detailing the experimental protocol. Gene expression, proliferation and
864 migration of tenocytes of all five species (human, mouse, rat, sheep, horse) were compared
865 under standard culture conditions (healthy control) as well as under transient (24 h) and
866 constant exposure to inflammatory stimuli (10 ng/ml IL1β and 10 ng/ml TNFα). After 24-hour
867 exposure to inflammation, the transient inflammation group received fresh culture medium,
868 while for the constant inflammation group fresh medium was again supplemented with
869 inflammatory factors.
870
871
872 Figure 3 : The proliferation capacity (A-C) of tenocytes from 5 different mammalian species
873 (mouse, rat, sheep, horse, and human) under healthy (Ctr/A), transient (TI/B) and constant
874 (CI/C) inflammatory condition is illustrated as fold increase over the course of 2 days (indicated
875 as mean ± SEM calculated from three biological replicates). For pairwise comparisons and
876 significance values see table 1.
877 A wound healing assay (D-F) was used to determine the migratory capacity of tenocytes from
878 five different mammalian species under healthy (D), transient (E) and constant (F) inflammatory
879 conditions (indicated as mean ± SEM calculated from three biological replicates). For pairwise
880 comparisons and significance values see table 1.
881
882
883 Figure 4: Scatter dot plots showing COL1, SCX, MMP1, MMP13, COX2 and IL6 gene expression
884 (presented as log2) of healthy tenocytes and tenocytes exposed to inflammatory stimuli for 24h
885 (transient inflammation) or continuously (constant inflammation) in different species (the black
886 lines indicate the respective means). Gene expression in the inflammatory conditions is shown
887 relative to the healthy tenocytes. Each dot represents a different biological replicate.
888 Differences were evaluated using ANOVA with Tukey HSD test, *p<0,05; **p<0,01; ***p<0,001
889
890
891 Figure 5: Plot of PC1 (explaining 36% of the variance) vs. PC3 (explaining 14% of the variance) of
892 a Principal Component Analysis of gene expression values. Species are colour coded, conditions
893 (healthy, transiently inflamed, and continuously inflamed) are differentiated by symbols. Each
894 dot represents a different biological replicate.
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899 **Tables**

900

901 Table 1: The first column corresponds to the healthy situation, the second and third to
902 transiently and constantly inflamed, respectively. First rows (human) correspond to tests for
903 non-zero slopes of the proliferation and migration curves in humans, i.e., either non-zero
904 proliferation or non-zero gap closure in humans. Second to fifth rows (different animal species)
905 correspond to tests of differences of the animal models to humans in the slopes of the
906 proliferation and migration curves of tenocytes. Calculations used an ANCOVA. Means,
907 standard errors and p-values are reported. To correct for multiple testing with four
908 comparisons using Bonferroni, the nominal significance levels (0.05, 0.01, and 0.001) are set to
909 the corrected levels (0.0125, 0.002, 0.0002, respectively). P-values are marked with stars from *
910 (significant) to *** (highly significant) using this correction.

911

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912

SPECIES		HEALTHY			TRANSIENT INFLAMMATION			CONSTANT INFLAMMATION		
		prolif. slope	*Std. error*	*p-value*	*slope: diff. to healthy*	*Std. error*	*p-value*	*slope: diff. to healthy*	*Std. error*	*p-value*
PROLIFERATION	**Human**	**0.1027**	**0.0168**	**1.75e-09 *****	-0.0167	0.0237	0.4801	0.0089	0.0237	0.7074
		slope: diff. to human	*St. error*	*p-value*	*slope: diff. to human*	*St. error*	*p-value*	*slope: diff. to human*	*St. error*	*p-value*
	Mouse	**-0.1250**	**0.0237**	**1.95e-07 *****	0.0508	0.0335	0.1301	-0.0301	0.0335	0.3691
	Rat	**0.0933**	**0.0237**	**9.35e-05 *****	-0.0038	0.0335	0.9103	0.0420	0.0335	0.2103
	Sheep	**0.1169**	**0.0237**	**1.10e-06 *****	0.0585	0.0335	0.0815	**-0.0878**	**0.0335**	**0.00900 ***
	Horse	**0.3000**	**0.0237**	**< 2e-16 *****	0.0233	0.0335	0.4868	-0.0640	0.0335	0.0568
		migration slope	*Std. Error*	*p-value*	*slope: diff. to healthy*	*St. error*	*p-value*	*slope: diff. to healthy*	*St. error*	*p-value*
MIGRATION	**Human**	**-0.0792257**	**0.0032**	**< 2e-16 *****	**0.0277**	**0.0045**	**2.76e-09 *****	**0.0475**	**0.0045**	**< 2e-16 *****
		slope: diff. to human	*Std. Error*	*p-value*	*slope: diff. to human*	*St. error*	*p-value*	*slope: diff. to human*	*St. error*	*p-value*
	Mouse	**0.0311503**	**0.0045**	**3.07e-11 *****	-0.0075	0.0064	0.2452	-0.0156	0.0064	0.0154
	Rat	**0.0001854**	**0.0046**	**0.9155**	-0.0063	0.0064	0.3287	0.0035	0.0065	0.5938
	Sheep	**0.0185550**	**0.0045**	**5.46e-05 *****	**-0.0188**	**0.0064**	**0.00374 ***	-0.0112	0.0064	0.0811
	Horse	**0.0132563**	**0.0045**	**0.00375 ****	-0.0123	0.0064	0.0567	-0.0140	0.0064	0.0302

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917 Table 2: Mean difference to human and p-values of the gene expression calculated with ANOVA
918 of healthy, transiently inflamed and constantly inflamed tenocytes of the four animal model
919 species. Significant p-values (Tukey HSD correction) and the matching mean differences in gene
920 expression to humans are indicated in bold.
921

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			SCX	TNC	TNM	COL1	COL3	COL5	MMP1	MMP3	MMP13	IL6	COX2	NFKB	P53	ALP	FAK
Healthy	mouse	diff	2.65	-0.74	-1.05	-3.03	2.54	-2.17	-9.34	9.45	8.50	4.65	9.40	-0.20	-0.30	3.14	-0.25
		adj p	0.230	0.811	0.951	0.038	0.122	0.609	0.064	0.002	0.000	0.005	0.000	0.999	0.961	0.032	0.995
	rat	diff	5.28	2.84	-1.37	3.95	3.80	4.62	-2.97	10.24	5.24	0.54	11.30	3.00	4.47	1.85	4.72
		adj p	0.008	0.014	0.882	0.008	0.015	0.067	0.851	0.001	0.013	0.978	0.000	0.053	0.000	0.287	0.000
	sheep	diff	2.39	3.79	-3.52	-7.91	-0.50	0.41	-4.71	9.12	9.47	-0.16	13.61	-0.72	-0.36	-0.14	3.95
		adj p	0.313	0.002	0.201	0.000	0.981	0.998	0.539	0.003	0.000	1.000	0.000	0.931	0.927	1.000	0.001
	horse	diff	1.49	-0.24	-3.11	-4.72	0.41	1.71	-2.93	1.94	5.37	-3.67	15.65	3.79	3.52	-1.11	2.11
		adj p	0.711	0.996	0.296	0.002	0.991	0.780	0.857	0.793	0.012	0.022	0.000	0.014	0.000	0.720	0.055
Transient inflammation	mouse	diff	-1.22	-0.69	-2.59	-2.62	-3.06	-3.07	1.23	-5.08	-4.96	-4.02	-4.15	-1.24	-0.59	0.46	-0.46
		adj p	0.515	0.870	0.048	0.177	0.017	0.010	0.984	0.011	0.001	0.001	0.000	0.180	0.803	0.990	0.963
	rat	diff	-2.94	-1.42	-2.85	-2.93	-4.43	-3.57	-0.66	-3.22	-1.56	-0.98	-4.84	-0.01	-0.34	-1.54	-1.48
		adj p	0.019	0.342	0.029	0.114	0.001	0.004	0.999	0.124	0.412	0.537	0.000	1.000	0.967	0.563	0.299
	sheep	diff	-0.59	-0.20	0.59	-0.12	-1.84	-1.49	0.39	-6.54	-4.19	-2.83	-6.20	-0.52	0.16	1.38	1.31
		adj p	0.930	0.998	0.937	1.000	0.189	0.291	1.000	0.002	0.004	0.007	0.000	0.841	0.998	0.651	0.404
	horse	diff	-0.77	1.44	-0.34	1.40	-0.36	-0.95	3.97	-0.32	0.15	3.25	-6.63	-0.16	1.38	2.22	0.98
		adj p	0.840	0.330	0.992	0.691	0.989	0.671	0.498	0.999	1.000	0.003	0.000	0.997	0.152	0.249	0.658
Constant inflammation	mouse	diff	-1.23	-0.51	-3.33	-1.89	-3.30	-2.52	1.64	-4.82	-3.41	-4.54	-3.96	-1.33	-0.09	2.83	0.22
		adj p	0.911	0.949	0.166	0.361	0.003	0.501	0.977	0.014	0.209	0.021	0.094	0.586	1.000	0.093	0.998
	rat	diff	-3.27	-1.76	-4.86	-3.80	-7.16	-5.29	4.75	-1.96	3.26	1.13	-1.71	0.13	0.18	-1.09	-1.35
		adj p	0.238	0.177	0.028	0.020	0.000	0.039	0.509	0.490	0.241	0.866	0.727	1.000	0.999	0.797	0.412
	sheep	diff	-2.01	-0.63	-1.41	0.56	-1.81	-2.94	1.51	-6.73	-2.93	-0.42	-7.68	-0.18	0.19	1.24	1.48
		adj p	0.653	0.900	0.822	0.975	0.111	0.366	0.983	0.001	0.328	0.996	0.002	1.000	0.999	0.719	0.333
	horse	diff	-3.10	2.28	-1.74	1.37	-0.39	-2.04	3.59	-0.24	1.49	5.53	-8.36	-0.63	0.95	3.18	0.58
		adj p	0.280	0.059	0.693	0.637	0.972	0.679	0.733	1.000	0.839	0.006	0.001	0.949	0.678	0.054	0.932

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922 Table 3: Mahalanobis distances of the four model species to humans for all genes combined
923 under healthy, transiently and constantly inflamed conditions as well as for the different
924 functional gene groups: tenogenic markers, collagens, MMPS and inflammatory mediators.
925

	Mouse	**Rat**	**Sheep**	**Horse**
Mahalanobis distances all genes per condition				
Healthy	20.01	27.44	24.78	23.05
Transient Inflammation	13.49	18.66	10.62	9.59
Constant Inflammation	10.50	17.45	8.94	12.97
Mahalanobis distances all conditions per group of genes				
SCX, TNC, TNMD	5.45	12.21	10.54	6.88
COL1, COL3, COL5	8.28	13.73	12.88	8.45
MMP1, MMP3, MMP13	8.53	11.27	8.60	4.65
COX2, IL6,	15.82	14.25	18.51	17.77

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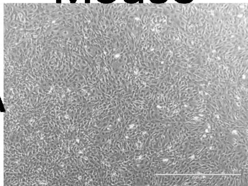
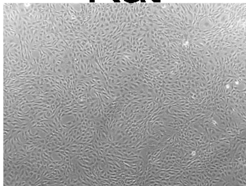
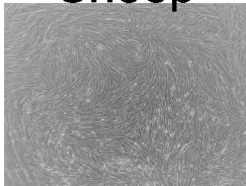

Mouse
A

Rat

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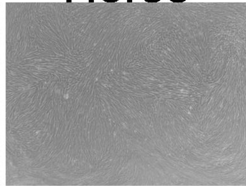
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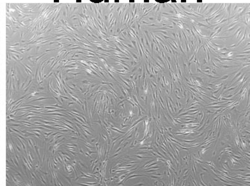
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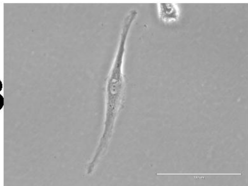
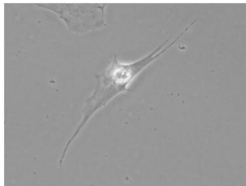
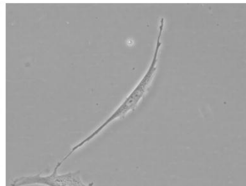
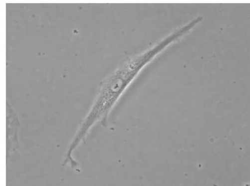
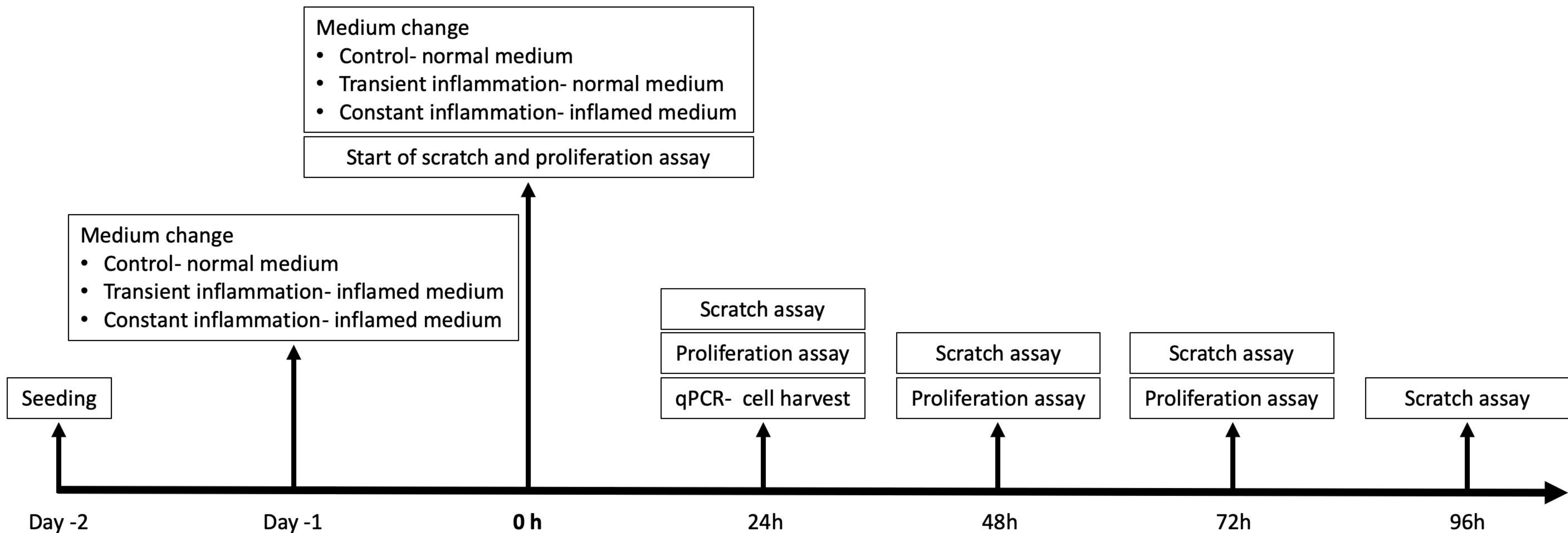
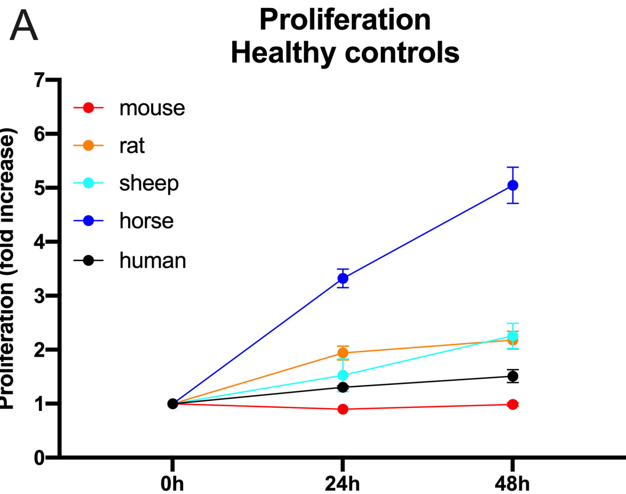
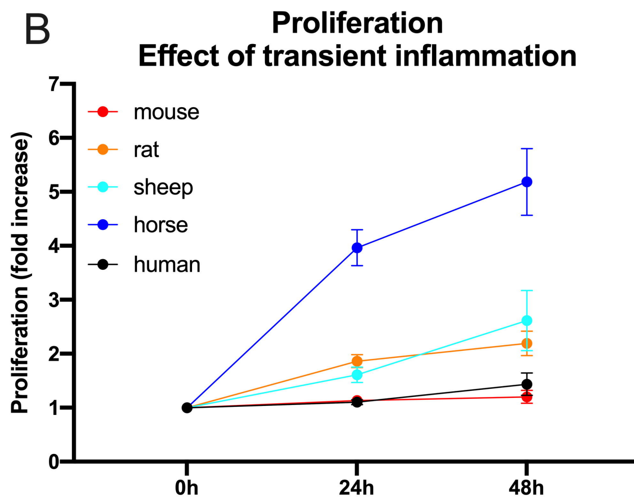

B

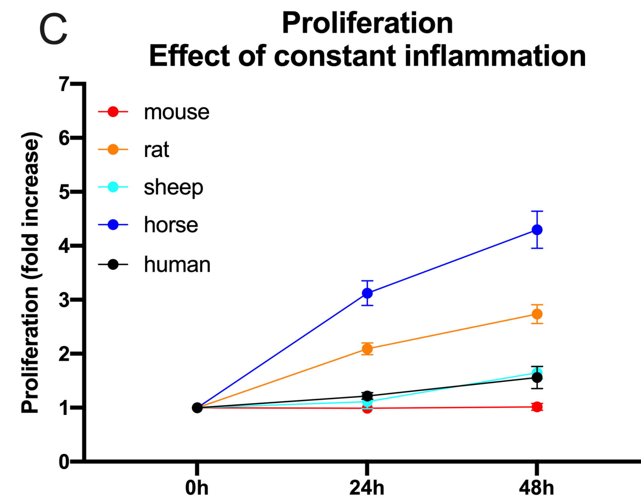




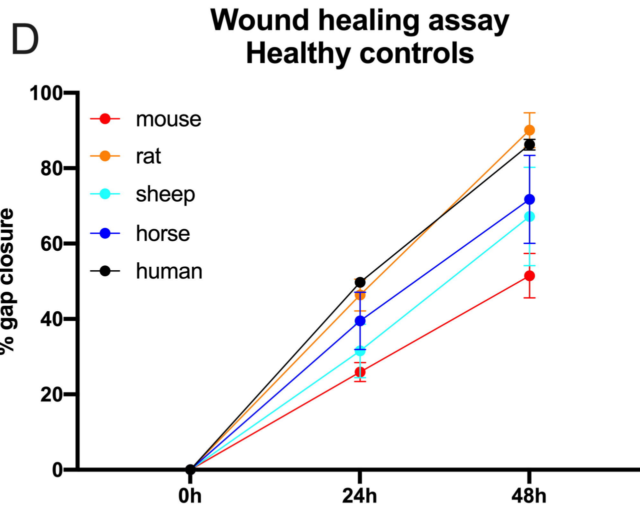


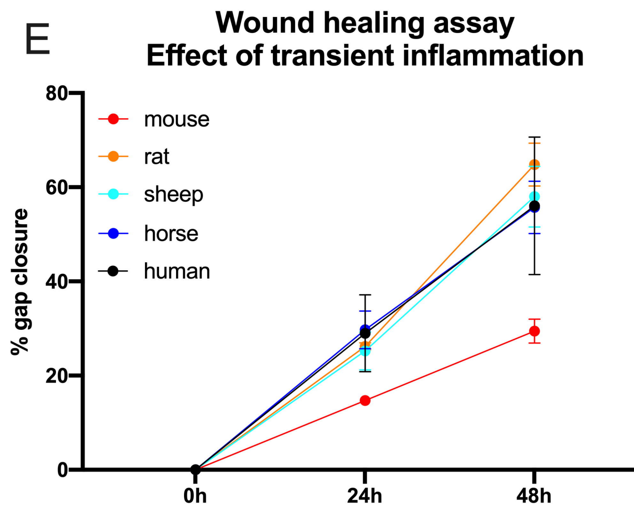
Medium change
• Control- normal medium
• Transient inflammation- normal medium
• Constant inflammation- inflamed medium
Start of scratch and proliferation assay
Medium change
• Control- normal medium
• Transient inflammation- inflamed medium
• Constant inflammation- inflamed medium
Scratch assay
Proliferation assay
qPCR- cell harvest
Scratch assay
Proliferation assay
Scratch assay
Proliferation assay
Seeding
Scratch assay
Day -2
Day -1
0 h
24h
48h
72h
96h

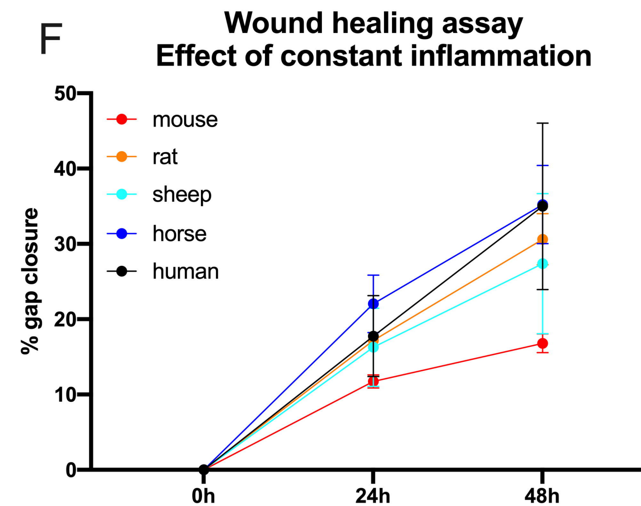

A
Proliferation
Healthy controls
Proliferation (fold increase)
mouse
rat
sheep
horse
human
0h
24h
48h


B
Proliferation
Effect of transient inflammation
Proliferation (fold increase)
mouse
rat
sheep
horse
human
0h
24h
48h

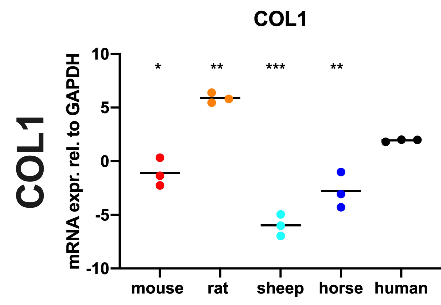

C
Proliferation
Effect of constant inflammation
Proliferation (fold increase)
mouse
rat
sheep
horse
human
0h
24h
48h

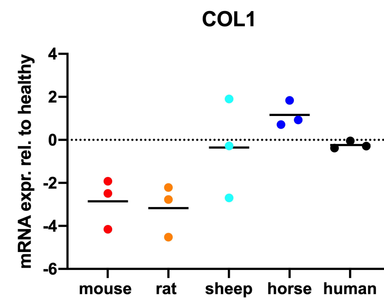

D
Wound healing assay
Healthy controls
% gap closure
mouse
rat
sheep
horse
human
0h
24h
48h

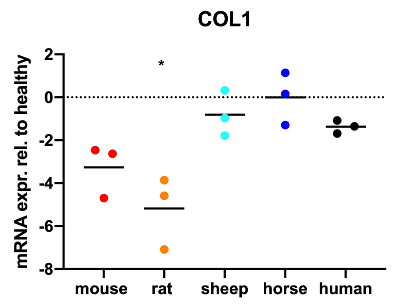

E
Wound healing assay
Effect of transient inflammation
% gap closure
mouse
rat
sheep
horse
human
0h
24h
48h

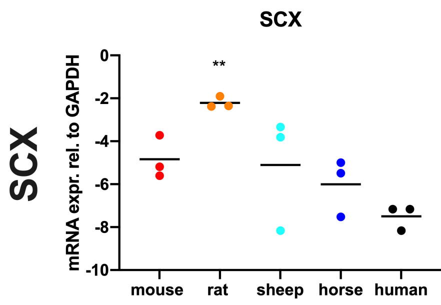

F
Wound healing assay
Effect of constant inflammation
% gap closure
mouse
rat
sheep
horse
human
0h
24h
48h

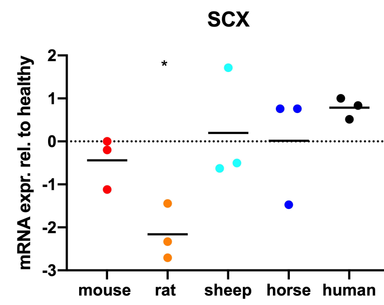
Healthy **Transient Inflammation** **Constant Inflammation**

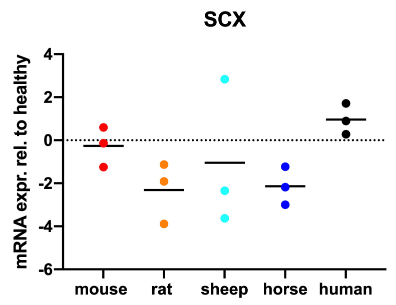


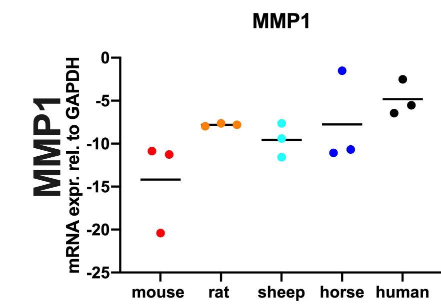


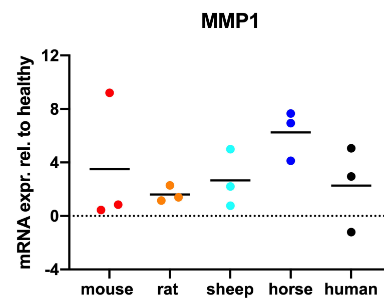


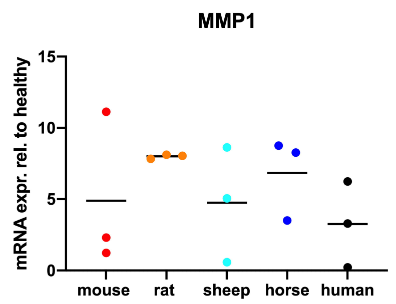


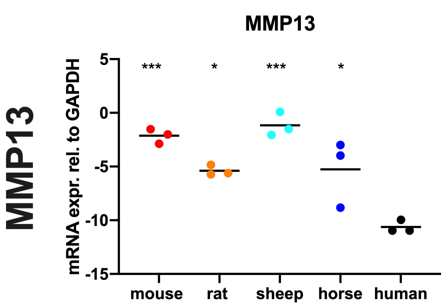


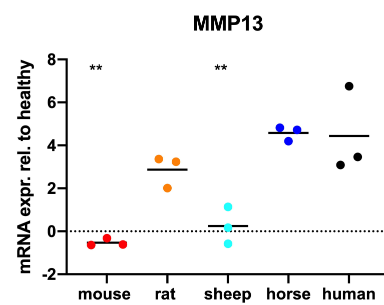


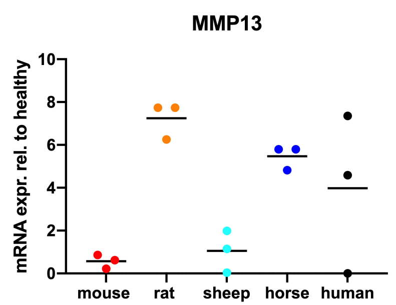


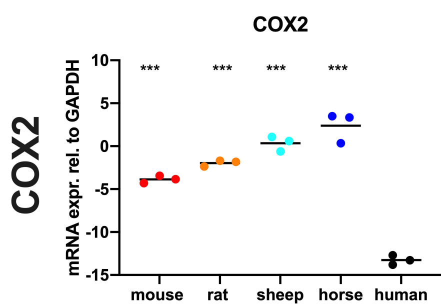


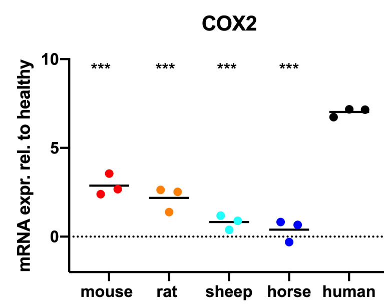


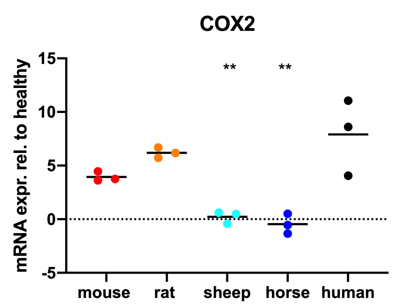


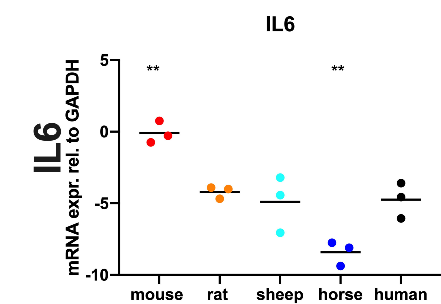


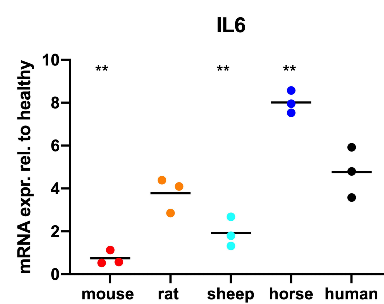


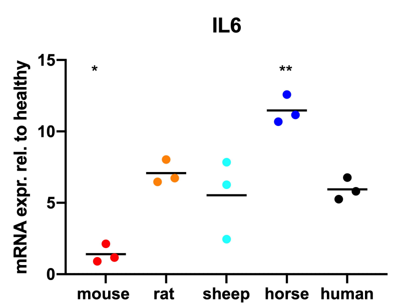


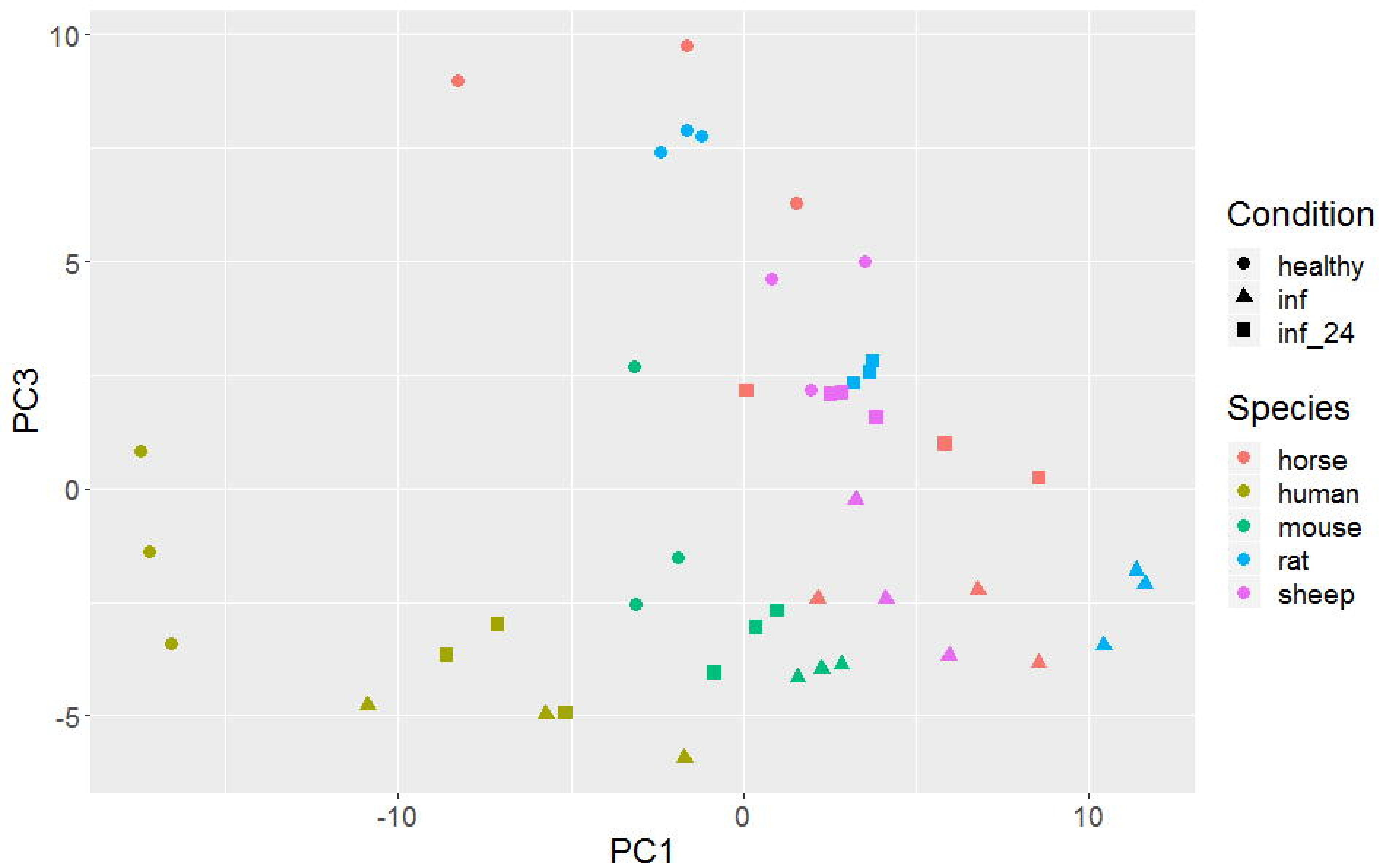












10
5
0
-5
-10
0
10
PC1
PC3
Condition
healthy
inf
inf_24
Species
horse
human
mouse
rat
sheep