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## Maternal age affects equine Day 8 embryo gene expression both in trophoblast and inner cell mass

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22 *Ethics approval and consent to participate*

23 The experiment was performed at the experimental farm of IFCE (research agreement C1903602 valid  
24 until March 22, 2023). The protocol was approved by the local animal care and use committee (“Comité  
25 des Utilisateurs de la Station Expérimentale de Chamberet”) and by the regional ethical committee  
26 (“Comité Régional d’Ethique pour l’Expérimentation Animale du Limousin”, approved under N° C2EA -  
27 33 in the National Registry of French Ethical Committees for animal experimentation) under protocol  
28 number APAFIS#14963-2018050316037888 v2. All experiments were performed in accordance with  
29 the European Union Directive 2010/63EU.

30

31 *Consent for publication*

32 Not applicable

33

34 *Availability of data and materials*

35 The RNA sequencing data supporting the conclusions of this article are available in the GEO  
36 repository, [accession: GSE162893;  
37 <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE162893>].

38

39 *Competing interests*

40 The authors declare that they have no competing interests.

41

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46

47 *Author contribution statement*

48 PCP obtained the funding. PCP and VD conceived the project. LW, PCP, and VD supervised the study.  
49 ED, CD, CA, ND, NP, LW, VD and PCP adapted the methodology for the project. ED, CD, CA, YJ, JAR, and  
50 LW performed the experiments. CD, CA, ND, NP, MD and LW provided the resources. ED, LJ, YJ and RL  
51 performed data curation. ED and LJ analysed the data. ED wrote the original draft. All authors read,  
52 revised, and approved the submitted manuscript.

53

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63

64 **Abstract**

65 **Background:** Increased embryo loss as mares become older is a major consideration for breeders as  
66 older animals are currently used for reproduction in the equine industry. Lower embryo quality has  
67 been pointed out as partly responsible for this reduced fertility. Here, the effect of mare's age on  
68 blastocysts' gene expression was explored. Day 8 post ovulation embryos were collected by uterine  
69 flushing from multiparous young (YM, 6-year-old, N = 5) and older (OM, > 10-year-old, N = 6) non-

70 nursing Saddlebred mares, inseminated with the semen of one stallion. Pure (TE\_part) or inner cell  
71 mass enriched (ICMandTE) trophoblast were obtained by embryo bisection and paired end, non-  
72 oriented RNA sequencing (Illumina, NextSeq500) was performed on each hemi-embryo. To  
73 discriminate gene expression in the ICM from that in the TE, deconvolution (DeMixT R package) was  
74 used on the ICMandTE dataset. Differential expression was analyzed (DESeq2) with embryo sex and  
75 diameter as cofactors using a false discovery rate < 0.05 cutoff. Functional annotation and classification  
76 of differentially expressed genes and gene set enrichment analysis were also performed.

77 **Results:** Maternal aging did not affect embryo recovery rate, embryo diameter nor total RNA quantity  
78 but modified gene expression in equine D8 embryos. The expression of genes in the ICM seemed to be  
79 more altered by maternal aging than in the TE. In both compartments, the expression of genes involved  
80 in mitochondrial function, translation and transcription due to chromatin modification were disturbed  
81 by maternal age. Mitosis, signaling and adhesion pathways and embryo development were particularly  
82 decreased in the ICM hemi-embryos from old mares. Finally, in TE, ion movement related genes were  
83 affected.

84 **Conclusions:** This is the first study showing an effect of maternal age on gene expression in the equine  
85 blastocyst at Day 8 post ovulation. Maternal age, even for mares as young as 10 years old, disturbs  
86 mitosis, translation and transcription, cell signaling and adhesion as well as mitochondrial function and  
87 cell commitment of horse embryos. These perturbations may affect further embryo development and  
88 contribute to decreased fertility due to aging.

89

90

91

## 92 **Keywords**

93 horse; aging; blastocyst; methylation; mitochondria; mitosis; cell commitment

94

## 95 **Background**

96 Unlike other farm animals, the main purpose for the use of horses is athletic performance and leisure.

97 In many cases, mares produce foals regularly to remain profitable after their sport career so that their  
98 reproductive career may last until advanced age, even though mares' fertility declines with age [1].

99 Indeed, in Thoroughbreds, pregnancy rates per estrous at Day 42 post-ovulation were shown to be  
100 reduced from 63.0% in young mares to 58.5% in mares older than 9 , and to reach 43.8% in mares older  
101 than 18 [2]. One study on 535,746 fertility records in mixed breeds in France showed that if a 10% of  
102 fertility decline is chosen as cutoff value for culling breeding mares, then mares older than 8 should  
103 not be bred [3].

104 Embryo collections and transfers at various developmental stages have been performed to determine  
105 at what stage embryos are affected and whether embryo or uterine quality is responsible for the  
106 fertility decline. Uterine blood flow during the luteal phase (Day 12-20 post ovulation) was reduced in  
107 mares >15 years old compared to mares <6 years old [4]. Moreover, at 2 days post ovulation, using  
108 oviductal flushes, the fertilization rate was similar between young and fertile mares vs old and  
109 subfertile mares (respectively, 10/14 vs 11/14 embryos recovered per group on Day 2). In contrast, in  
110 the same study, using the same mares on another estrus period, a significantly higher pregnancy rate  
111 at 14 days post ovulation was observed in young and fertile mares (respectively, 12/15 vs 3/15  
112 pregnant mares on Day 14 by ultrasonography) [5]. Moreover, in practice, embryo loss is generally  
113 higher between the first (14 - 22 days) and the second (42 - 45 days) pregnancy check in old compared  
114 to young mares [2,6,7]. Using a ROC analysis based on 11 years of data from nursing Thoroughbred  
115 mares in Brazil, the cutoff value for embryo mortality to reach above average values was calculated to  
116 be 10 years of age [7]. In addition, Day 4 embryos collected from old and subfertile mares and directly  
117 transferred in synchronized young and fertile mares, were shown to result in lower pregnancy rates at  
118 14 days post ovulation compared to embryos from young and fertile mares transferred in the same  
119 recipient group. This was true even when only good quality embryos were considered [8]. Thus,

120 embryo loss seems to be the principal cause of the fertility decline in old mares, even if uterine  
121 degeneration is also observed in old mares.

122 In terms of embryo morphology, the effect of maternal age is not so clear, as studies demonstrated a  
123 reduction of embryo size in old mares while others did not detect any effect of maternal aging on  
124 embryo size. In these studies, however, the developmental stage was not uniform, which might lead  
125 to these disparities [5,8–15]. For stages of development that take place in the oviduct, *i.e.*, from  
126 fertilization to the late morula stage, one study on embryos collected *in vivo* on Day 1.5 post ovulation  
127 showed that embryos have the same number of cells but a poorer morphology grade (uniformity of  
128 cell size and texture, imperfections and abnormalities) when mares are older than 15 [11]. In addition,  
129 when mares were older than 20 years, Day 3 post ovulation embryos were shown to combine a poorer  
130 morphology grade with reduced cell number [11]. In contrast, in other studies, no difference according  
131 to maternal age was observed in terms of diameter or number, size, texture of blastomeres of embryos  
132 collected in oviducts on Days 2 and 4 post ovulation [5,8]. For later stages, the size of embryos collected  
133 on Days 7 to 10 post-ovulation, in commercial embryo transfer programs in Italy, was reduced in mares  
134 older than 16 years of age, without any morphological grade alteration [10]. In contrast, smaller  
135 embryos, with a higher incidence of morphological abnormalities, were collected when donors were  
136 subfertile and/or 18 years old mares compared to 4 years old nulliparous mares [12]. In embryos  
137 produced by intracytoplasmic sperm injection (ICSI), maternal age did not affect blastomere size, nor  
138 the presence of ooplasm fragments, nor membrane extrudates through zona pellucida at 24h post-  
139 ICSI [9]. Embryo cell number at the time of transfer (between 6 and 9 days post-ICSI), however, was  
140 higher in embryos collected from mares older than 20 [8].

141 In terms of functional potential, a reduced quantity of mitochondrial DNA, but not genomic DNA, has  
142 been reported in Day 7 embryos from mares older than 16 [16]. These data suggest that old mares'  
143 blastocysts have the same number of cells than those of younger mares, but with less mitochondria  
144 [16], which could be a cause or an effect of compromised embryo development in old mares.  
145 Moreover, embryos from mares older than 15 seem to be less mobile before implantation than those

146 from mares aged 5-6 [14]. This could indicate impaired embryo development, alteration of uterine  
147 contractions or both.

148 Altogether, these data indicate that the quality of embryos from older mares is reduced. Subsequently,  
149 dam age could also affect placental and fetal intrauterine and offspring post-natal growth and long-  
150 term health, in the context of the Developmental Origins of Health and Diseases (DOHaD) [17]. To our  
151 knowledge, the role of the embryo *per se*, however, has not yet been explored. Moreover, age and  
152 parity are generally positively correlated and their respective impact have not been determined yet.

153 The aim of this study was to determine the effect of maternal age in the mare on embryo gene  
154 expression at the blastocyst stage. Young (<10 years) and older (>10 years) multiparous mares were  
155 inseminated with the same stallion. Blastocysts were collected and bisected to separate the pure  
156 trophoblast (TE\_part) from the inner cell mass enriched hemi-embryo (ICMandTE). Gene expression  
157 was analyzed by RNA-seq in each compartment. Deconvolution was used to isolate gene expression of  
158 inner cell mass from trophoblast cells in ICMandTE part. Results shows that maternal age alters gene  
159 expression of both equine Day 8 trophoblast and inner cell mass.

160

## 161 **Materials and methods**

### 162 *Ethics*

163 The experiment was performed at the experimental farm of IFCE (research agreement C1903602 valid  
164 until March 22, 2023). The protocol was approved by the local animal care and use committee (“Comité  
165 des Utilisateurs de la Station Expérimentale de Chamberet”) and by the regional ethical committee  
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167 33 in the National Registry of French Ethical Committees for animal experimentation) under protocol  
168 number APAFIS#14963-2018050316037888 v2. All experiments were performed in accordance with  
169 the European Union Directive 2010/63EU.

170

171 *Embryo collection*

172 Thirty-one multiparous Saddlebred mares (French Anglo-Arabian and Selle Français breeds) were  
173 included in this study. Multiparous mares were defined as dams that had already foaled at least once.  
174 Mares were bred and raised in the “Institut Français du Cheval et de l’Equitation” experimental farm  
175 (Chamberet, France, 45° 34’55.17”N, 1°43’16.29”E, 442m). During the experimental protocol, mares  
176 were managed in herds in natural pastures 24h/day with free access to water. The experiments took  
177 place from April 1<sup>st</sup> to May 23<sup>rd</sup>, 2019. All mares remained healthy during this period.

178 Mares were allocated to 2 groups according to their age: young mares (YM, < 10 years, n = 11) and  
179 older mares (OM, ≥ 10 years, n = 20). The age threshold was defined in accordance with the literature  
180 [3,7]. Before experimentation, mare’s withers’ height was measured. During the experimentation,  
181 mares were weighted and mares’ body condition score (BCS, scale 1 to 5) was determined by one  
182 trained operator [18]. Mares’ characteristics are detailed in Table 1.

183

184 Table 1: Mares’ characteristics at embryo collection time.

Characteristics	Young mares (YM) (n=11)	Old mares (OM) (n=20)
Breed	AA n = 1; SF n =9; SB n=1	AA n = 13; SF n = 7
Age (in years)	6.00 ± 0.00	11.95 ± 2.11
Parity (number of foalings)	1.00 ± 0.00	3.20 ± 1.15
Weight (in kg)	536.62 ± 44.12	589.35 ± 57.34
BCS (scale 1-5)	2.16 ± 0.28	2.61 ± 0.32
Withers’ height (in cm)	157.95 ± 3.74	161.21 ± 4.23

185 All mares were multiparous.

186 AA: Anglo Arab or Anglo-Arabian type; SF: Selle Français; SB: Saddlebred; BCS: Body Condition Score.

187 Age, parity, weight, BCS and height are presented as mean ±SD

188

189 The mares' estrous period was monitored routinely by ultrasound with a 5MHz trans-rectal transducer.  
190 During estrus, ovulation was induced with a single injection of human chorionic gonadotropin (i.v.; 750  
191 - 1500IU; Chorulon® 5000; MSD Santé animale, France) as soon as one ovarian follicle > 35mm in  
192 diameter was observed, together with marked uterine edema. Ovulation usually takes place within  
193 48h, with > 80% occurring 25 to 48h after injection [19,20]. At the same time, mares were inseminated  
194 once with fresh or refrigerated semen containing at least 1 billion motile spermatozoa from a single  
195 fertile stallion. Ovulation was confirmed within the next 48 hours by ultrasonography.

196 Embryos were collected by non-surgical uterine lavage using prewarmed (37°C) lactated Ringer's  
197 solution (B.Braun, France) and EZ-Way Filter (IMV Technologies, France) 10 days after insemination,  
198 i.e. approximately 8 days post ovulation. Just after embryo collection, mares were treated with  
199 luproliol an analogue of prostaglandin F2 $\alpha$  (i.m; 7.5 mg; Prosolvin, Virbac, France).

200

#### 201 *Embryo bisection and RNA extraction*

202 Collected embryos were immediately observed using a binocular magnifying glass and photographed  
203 with a size standard. Embryo diameter was subsequently determined on photographs using ImageJ®  
204 software (version 1.52a; National Institutes of Health, Bethesda, MD, USA). Embryos were washed 4  
205 times in commercially available Embryo holding medium (IMV Technologies, France) at 34°C and then  
206 bisected with a microscalpel under binocular magnifying glass to separate trophoblast part, at this  
207 stage composed of trophectoderm and endoderm (TE\_part) from inner cell mass (composed of  
208 epiblast cells) enriched hemi-embryos (ICMandTE) (Figure 1). Each hemi-embryo was transferred to an  
209 Eppendorf DNA LoBind tube (Eppendorf, Montesson, France) with extraction buffer (PicoPure RNA  
210 isolation kit, Applied Biosystems, France) and incubated for 30 min at 42°C prior to storage at - 80°C.  
211 RNA was extracted from each hemi-embryo following the manufacturer's instructions using PicoPure  
212 RNA isolation kit (PicoPure RNA isolation kit, Applied Biosystems, France), which included a DNase

213 treatment. RNA quality and quantity were assessed with the 2100 Bioanalyzer system using RNA 6000  
214 Pico or nano kit (Agilent Technologies, France) according to the manufacturer's instructions (Additional  
215 file 1).

216

### 217 *RNA sequencing*

218 Five nanograms of total RNA were mixed (Additional file 1) with ERCC spike-in mix (ThermoFisher  
219 Scientific, France) according to manufacturer recommendations. Messenger RNAs were reverse  
220 transcribed and amplified using the SMART-Seq V4 ultra low input RNA kit (Clontech, France) according  
221 to the manufacturer recommendations. Nine PCR cycles were performed for each hemi-embryo. cDNA  
222 quality was assessed on an Agilent Bioanalyzer 2100, using an Agilent High Sensitivity DNA Kit (Agilent  
223 Technologies, France). Libraries were prepared from 0.15 ng cDNA using the Nextera XT Illumina library  
224 preparation kit (Illumina, France). Libraries were pooled in equimolar proportions and sequenced  
225 (Paired end 50-34 pb) on NextSeq500 instrument, using a NextSeq 500 High Output 75 cycles kit  
226 (Illumina, France) (Additional file 1). Demultiplexing was performed with bcl2fastq2 version 2.2.18.12  
227 (Illumina, France) and adapters were trimmed with Cutadapt version 1.15 [21]. Only reads longer than  
228 10pb were kept.

229

### 230 *Embryo sexing*

231 Embryo sex was determined for each embryo in order to take in consideration embryo sex in the  
232 statistical analysis as sexual dimorphism was observed in blastocysts in other species [22].

233 The expression of *X Inactive Specific Transcript (XIST)* was analyzed. *XIST* is a long noncoding RNA from  
234 chromosome X responsible for X inactivation when two X chromosomes are present, meaning that  
235 only female embryos express *XIST*. *XIST* was shown to be expressed in equine female blastocysts on  
236 Day 8 post ovulation [23,24]. *XIST* is not annotated in the available equine Ensembl 3.0.99 or NCBI

237 annotation. Thus, a *de novo* transcript assembly was made using StringTie version 2.1.2 [25] following  
238 manufacturer instructions to identify *XIST* in equine embryo samples (Additional file 1). The genome  
239 annotation file output was aligned on Ensembl 99 EquCab3.0 assembly using IGV version 2.7.2 [26]. As  
240 *XIST* position and length on X chromosome has been previously determined (Pablo Ross, personal  
241 communication, [23]) and as *XIST* is annotated in humans, the most similar contig was selected. The  
242 obtained *XIST* sequence was 22,600 bp long and composed of 8 exons and 7 introns. *XIST* sequence  
243 was added to FASTA files from Ensembl 99 EquCab3.0 assembly and to the genome annotation file  
244 from Ensembl 99 EquCab3.0 assembly as a unique fictitious chromosome with one gene composed of  
245 one transcript.

246 *XIST* expression differed between individuals but was relatively close in quantification between related  
247 hemi-embryos. Therefore, seven embryos were determined as females (4 in the YM group and 3 in the  
248 OM group) while 4 were considered as males (1 in the YM group, and 3 in the OM group).

249

#### 250 *RNA mapping and counting*

251 Alignment was performed using STAR version 2.6 [27] (2-pass mapping with the genome annotation  
252 file using the following parameters: --alignSoftClipAtReferenceEnds No --alignEndsType EndToEnd --  
253 alignIntronMax 86545 --alignMatesGapMax 86545) on previously modified Ensembl 99 EquCab3.0  
254 assembly and annotation. Genes were then counted with featureCounts [28] from Subreads package  
255 version 1.6.1 (parameters: -p -B -C -M) (Additional file 1).

256

#### 257 *Data analysis*

258 All statistical analyses were performed by comparing OM to YM (set as reference group) using R version  
259 4.0.2 [29] on Rstudio software version 1.3.1056 [30].

260

261 ***Embryo recovery rate, embryo diameter and total RNA content analysis***

262 Embryo recovery rates (ERR) were calculated as number of attempts with at least one embryo  
263 collected/total number of attempts. They were analyzed using the Exact Fisher test to determine if age  
264 influences embryo recovery rate.

265 For total RNA content analysis, as embryos were bisected without strict equality for each hemi-  
266 embryo, a separate analysis of ICMandTE and TE\_part RNA quantities would not be meaningful. Thus,  
267 ICMandTE and TE\_part RNA quantities were initially summed up and analyzed using a mixed linear  
268 model from nlme package version 3.1-148 [31], followed by 1000 permutations using PermTest  
269 function from pgirmess package version 1.6.9 [32]. Maternal age and embryo sex were considered as  
270 random effects.

271 Embryo diameter was analyzed with a fixed effects linear model of nlme package version 3.1-148 [31]  
272 including maternal age and embryo sex, followed by 1000 permutations using PermTest function from  
273 pgirmess package version 1.6.9 [32]. Variables were kept in models when statistically significant  
274 differences were observed. Differences were considered as significant for  $p < 0.05$ .

275 Moreover, the relation between embryo size and total RNA quantity/embryo was studied and  
276 represented using the nonlinear regression analysis (fitness to exponential growth equation) in  
277 GraphPad Prism software 8.0.1 for Windows (Graphpad Software, San Diego, California USA,  
278 [www.graphpad.com](http://www.graphpad.com)).

279

280 ***Deconvolution of gene expression in ICMandTE using DeMixT***

281 A filter on counts obtained with featureCounts was applied: all genes with more than 3 non null count  
282 values in at least one group (OM or YM) per hemi-embryo (ICMandTE or TE\_part) were conserved. The  
283 ICMandTE hemi-embryo was composed of both trophoblast and inner cell mass in unknown relative  
284 proportions. In order to estimate the relative gene expression of both cell types, the DeMixT R package

285 version 1.4.0 was used [33,34]. Starting from a dataset obtained from one cell type, DeMixT estimates  
286 the proportion of cells in the mixed samples and performs a deconvolution algorithm on gene  
287 expression. TE\_part (reference cells) and ICMandTE (mixed cells type) datasets were thus analyzed  
288 using the following options: niter=10; nbin=50; if.filter=TRUE; ngene.selected.for.pi=1500; ngene.  
289 Profile.selected=1500; nspikein=0. Since the deconvolution algorithm cannot be performed in the  
290 presence of null values, the value “1” was added to all count values in ICMandTE and TE\_part data  
291 tables. Moreover, variance in reference cell dataset (TE\_part) must not be null and gene with null  
292 variance had been removed prior to the deconvolution. Output datasets were DeMixT\_ICM\_cells and  
293 DeMixT\_TE\_cells, corresponding to the deconvoluted gene expression in ICM cells and TE cells of  
294 ICMandTE, respectively.

295

#### 296 ***Quality checks for deconvolution***

297 At the end of deconvolution, a quality check is automatically performed by the analysis: for every gene,  
298 if the difference of average of deconvoluted expression of reference cells in mixed samples (here, gene  
299 expression of TE cells in ICMandTE dataset, *i.e.*, DeMixT\_TE\_cells) and average of expression of  
300 reference cells (here, gene expression of TE cells in TE\_part) is  $> 4$ , then, the deconvolution for  
301 concerned genes is not reliable. Therefore, genes in this case are filtered out.

302 Moreover, outputs of DeMixT\_ICM\_cells vs DeMixT\_TE\_cells, DeMixT\_ICM\_cells vs TE\_part and  
303 ICMandTE vs TE\_part were compared with Deseq2 version 1.28.1 [35] to confirm that the  
304 deconvolution was effective at separating gene expression. Moreover, several genes proper to ICM  
305 and TE cells in equine embryos were identified using literature search [23]. Their raw and deconvoluted  
306 expressions were analyzed to check the reliability of deconvolution. Results of these analyses were  
307 represented through manually drawn Venn diagrams as well as principal component analysis graphics  
308 of individuals, using ggplot2 version 3.3.3 [36] and factoextra version 1.0.7 [37].

309

310 ***Maternal age comparison for gene expression***

311 For the comparison of maternal age, ICMandTE, DeMixT\_ICM\_cells and TE\_part datasets were used  
312 and a filter was applied on datasets: all genes with an average expression equal or above 10 counts in  
313 at least one group (OM or YM) per hemi-embryo (ICM or TE) were conserved. Differential analyses  
314 were performed with Deseq2 version 1.28.1 [35] with YM group as reference, without independent  
315 filtering and taking into account embryo diameter and sex in the model. Genes were considered  
316 differentially expressed (DEG) for FDR < 0.05 after Benjamini-Hochberg correction (also known as false  
317 discovery rate, FDR).

318 Equine Ensembl IDs were converted into Human Ensembl IDs and Entrez Gene names using gorth  
319 function in gprofiler2 package version 0.1.9 [38]. Genes without Entrez Gene names using gprofiler2  
320 were manually converted when Entrez Gene names were available, using Ensembl web search function  
321 [39].

322 Moreover, DEG were analyzed for classification, over- and under- representation (FDR < 0.05) using  
323 Fisher's exact test corrected with FDR and using the database Gene Ontology (GO) Biological Process  
324 complete on PANTHER version 16.0 web-based software [40]. Only the most specific subclass  
325 pathways, provided by PANTHER's automated hierarchy sorting, were conserved and represented.

326

327 ***Gene set enrichment analyses (GSEA)***

328 The count values were log transformed using RLOG function of DESeq2 version 1.28.1. Gene set  
329 enrichment analyses (GSEA) were performed on expressed genes using GSEA software version 4.0.3  
330 (Broad Institute, Inc., Massachusetts Institute of Technology, and Regents of the University of  
331 California) [41,42] to identify biological gene sets disturbed by age. GSEA was performed using the  
332 following parameters: 1000 gene set permutations, weighted enrichment analysis, gene set size  
333 between 15 and 500, signal-to-noise metrics. Molecular Signatures Database [43] version 7.1 (C2:

334 KEGG: Kyoto Encyclopedia of Genes and Genomes, C5: BP: GO biological process) were used to identify  
335 most perturbed pathways. Pathways were considered significantly enriched for  $FDR < 0.05$ . When  
336 normalized enrichment score (NES) was positive, the gene set was enriched in the OM group while  
337 when NES was negative, the gene set was enriched in the YM group.

338 Enriched terms from GO BP and KEGG database were represented using SUMER analysis from SUMER  
339 R package version 1.1.5 and using FDR q-values [44]. First, SUMER analysis reduces redundancy using  
340 weighted set cover algorithm and then clusters the results using affinity propagation algorithm. The  
341 weighted set cover algorithm performs a selection of the fewest number of gene sets that include all  
342 genes associated with the enriched sets, giving priority for the most significant terms. Then, the affinity  
343 propagation algorithm groups similar gene sets and identifies one representative gene set. This  
344 algorithm will repeat this clustering to obtain the most representative gene sets. Results were  
345 represented with graph modified using Cytoscape version 3.8.2 [45]. Gene sets were represented by  
346 nodes and the gene set size was represented by the size of the node. Node shape represented the  
347 gene set database (GO BP or KEGG). Blue nodes represented gene sets enriched in YM ( $NES < 0$ ) while  
348 red nodes represented gene sets enriched in OM ( $NES > 0$ ). Edge width represents the level of  
349 connection between representative gene sets (thinner edges represent the first clustering while  
350 thicker edges represent the second clustering of the affinity propagation algorithm)

351

## 352 **Results**

### 353 *Embryo rates, diameter, total RNA content and quality*

354 Altogether, 36 uterine flushings were performed (12 in YM and 24 in OM, 5 mares being flushed twice).  
355 No twin embryos were obtained. Embryo recovery rate was 42% and 25% in YM and OM, respectively  
356 and did not differ between groups ( $p = 0.45$ ). All embryos were expanded blastocysts grade I or II  
357 according to the embryo classification of McKinnon and Squires [46].

358 Embryo diameter ranged from 409  $\mu\text{m}$  to 2643  $\mu\text{m}$ , with no effect of group on embryo diameter ( $p =$   
359 0.97). RNA yield per embryo ranged from 16 ng to 2915.5 ng and was not related to mares' age ( $p =$   
360 0.92). There is a clear exponential relation between RNA yield/embryo and embryo size (Figure 2).

361 The median RNA Integrity Number (RIN) was 9.8 (8.7 - 10 range). Between 29.4 and 69.5 million reads  
362 per sample were obtained after trimming. On average, 83.61% of reads were mapped on the modified  
363 EquCab 3.0 using STAR and 66.66% of reads were assigned to genes by featureCounts.

364

365 *Deconvolution of gene expression to discriminate ICM and TE gene expression in ICMandTE*  
366 *hemi-embryos*

367 After keeping genes with less than 3 non null count values in at least one group (OM or YM) per hemi-  
368 embryo (ICMandTE or TE\_part), 16,741 genes were conserved for deconvolution. In addition, twenty-  
369 seven genes were removed because their variance was null in the TE\_part. However, for these genes,  
370 the mean counting in ICMandTE samples was above 10 counts, excepted for one which had a mean  
371 expression of 34 counts. On the other hand, this gene was only expressed in 5 ICMandTE over the 10  
372 counts threshold.

373 During the analysis, 3 additional genes were excluded because the deconvolution quality for these  
374 genes was not sufficient. Therefore, at the end of deconvolution algorithm, 16,711 genes were  
375 available for differential analysis.

376 Before deconvolution, 341 genes were differentially expressed ( $\text{FDR} < 0.05$ ) between the ICMandTE  
377 and the TE\_part (Figure 3A). After deconvolution, comparison between DeMixT\_ICM\_cells and  
378 DeMixT\_TE\_cells yielded 7,565 differentially expressed genes while the comparison DeMixT\_ICM\_cells  
379 vs TE\_part yielded 6,085 differentially expressed genes, with 5,925 in common (72%). Moreover,  
380 except one of the initially 341 differentially expressed genes before deconvolution, were also identified  
381 as differentially expressed in both post-deconvolution analyses. On the PCA graphic of individuals,  
382 ICMandTE and TE\_part were not really separated before deconvolution (Figure 3B). DeMixT\_TE\_cells  
383 and TE\_part were partly superposed, suggesting that datasets before and after deconvolution have a

384 similar global gene expression; whereas the DeMixT\_ICM\_cells group is clearly separated from both  
 385 on Axis 2 (14.3% of variance), indicating that the deconvolution effectively enabled the separation of  
 386 gene expression in the two cell types.

387 Six of the 12 genes previously identified as more expressed in ICM [23] were also more expressed in  
 388 ICMandTE vs TE\_part comparison (Table 2). After deconvolution (comparison DeMixT\_ICM\_cells vs  
 389 TE\_part), 3 more genes previously identified were also differentially expressed. However, for two of  
 390 them (*POU Class 5 Homeobox 1*, *POU5F1* and *Undifferentiated Embryonic Cell Transcription Factor 1*,  
 391 *UTF1*), their expression was increased in TE\_part after deconvolution. In TE, any genes previously  
 392 identified were differentially expressed in the comparison ICMandTE vs TE\_part, *i.e.*, before  
 393 deconvolution. After deconvolution, the expression of 2 genes was increased in TE\_part compared to  
 394 DeMixT\_ICM\_cells.

395

396 Table 2: Comparison of selected genes expression before and after deconvolution

	Gene name	Ensembl ID	ICMandTE vs TE_part		DeMixT_ICM_cells vs TE_part	
			log2FC from DeSeq2	padj	log2FC from DeSeq2	padj
<b>ICM</b>	<b>SOX2</b>	ENSECAG00000010653	4.49	0.02	6.49	1.7E-06
	<b>NANOG</b>	ENSECAG00000012614	4.24	1.0E-07	5.98	2.4E-15
	<b>SPP1</b>	ENSECAG00000017191	4.02	8.4E-09	5.74	5.1E-16
	<b>LIN28B</b>	ENSECAG00000020994	3.23	1.3E-10	5.00	4.7E-22
	<b>SMARCA2</b>	ENSECAG00000024187	0.62	0.97	1.25	0.07
	<b>POU5F1 (OCT4)</b>	ENSECAG00000008967	0.60	0.009	-2.04	4.6E-07
	<b>ID2</b>	ENSECAG00000008738	0.44	0.29	1.24	9.0E-05
	<b>DNMT3B</b>	ENSECAG00000012102	0.35	0.24	0.8	0.06
	<b>DPPA4</b>	ENSECAG00000013271	0.35	0.04	1.14	1.8E-05
	<b>SALL4</b>	ENSECAG00000018533	0.10	1	0.60	0.01
	<b>KLF4</b>	ENSECAG00000010613	-0.04	1	-0.81	0.39
	<b>UTF1</b>	ENSECAG00000039888	-0.13	1	-2.21	7.3E-05

<b>TE</b>	<b>TFAP2A</b>	ENSECAG00000017468	-0.17	0.46	-0.30	0.004
	<b>CDX2</b>	ENSECAG00000027754	-0.14	0.77	-0.23	0.07
	<b>ELF3</b>	ENSECAG00000014608	-0.13	1	-0.19	0.27
	<b>GATA2</b>	ENSECAG00000016768	-0.13	1	-0.29	0.048
	<b>GATA3</b>	ENSECAG00000024574	-0.10	0.87	-0.05	0.64
	<b>TEAD4</b>	ENSECAG00000011303	-0.10	0.96	0.00	1
	<b>FREM2</b>	ENSECAG00000020410	0.07	1	0.19	0.44

397

398 Gene expressions were obtained from RNA of 11 equine embryos bisected in two hemi-embryos: one  
 399 part is composed only of trophoblast (TE), TE\_part, while the other part is composed of TE and inner  
 400 cell mass (ICM), ICMandTE. As it is impossible to estimate the proportion of each cells in ICMandTE,  
 401 deconvolution algorithm (package DeMixT) was used to estimate gene expression of these different  
 402 kind of cells. DeMixT\_ICM\_cells dataset is the deconvoluted gene expression of ICM cells from  
 403 ICMandTE. Log2 fold change (log2FC) and padj (adjusted p-value with Benjamini-Hochberg correction)  
 404 were obtained with Deseq2 package. TE\_part is the reference group in both analyses: when log2 fold  
 405 changes (log2FC) > 0, gene is more expressed in ICMandTE or DeMixT\_ICM\_cells while when log2FC <  
 406 0, gene is more expressed in TE\_part. Green is used to represent gene differentially expressed in the  
 407 present study. Orange is used to represent gens that have been previously identified as predominant  
 408 in the ICM [23] but which are identified here as predominant in the TE.

409

410 For further analysis, DeMixT\_ICM\_cells and TE\_part gene expression datasets are presented.  
 411 Nevertheless, the comparison of maternal age on ICMandTE dataset (graphics, differential gene  
 412 expression analysis and GSEA) was also performed and this analysis is available in Additional file 2, 3  
 413 and 4.

414

415 *Differential gene expression in deconvoluted ICM cells' genes expression according to maternal*

416 *age*

417 After filtering out of genes with an average expression < 10 counts/maternal age group/hemi-embryo,  
418 13,735 genes were considered as expressed in the ICM cells from OM or YM embryos including 255  
419 differentially expressed genes (227 downregulated and 28 upregulated in OM) (Figure 4A and  
420 Additional file 5). Only 218 and 12 genes out of the down- and upregulated genes, respectively, were  
421 associated to a known protein in human. These downregulated genes in ICM of embryos from OM  
422 mainly belonged to cellular process, metabolic process and biological regulation in GO biological  
423 process gene sets (Figure 4A). From downregulated genes in OM ICM, 84 pathways were statistically  
424 overrepresented in GO Biological process leading to 16 most specific subclass pathways according to  
425 hierarchy sorting by PANTHER (Figure 4B). Overrepresented pathways in downregulated genes in OM  
426 ICM were linked to cell division, embryo morphogenesis, histone methylation, protein modification  
427 processes and cytoskeleton organization.

428 Upregulated DEGs in OM ICM were mainly part of biological regulation, response to stimulus, cellular  
429 process, signaling and metabolic process in GO biological process gene sets (Figure 4A). No pathway  
430 was statistically overrepresented in upregulated genes in ICM of embryos from old mares using  
431 PANTHER overrepresentation testing.

432

#### 433 *Differential gene expression in pure TE part gene expression*

434 In the TE, 13,178 genes were considered as expressed in OM or YM. Fourteen were differentially  
435 expressed (Additional file 6) with 11 genes being downregulated and 3 being up regulated in OM  
436 (Figure 4A). Only 12 genes were associated to a known protein in human. Downregulated genes in OM  
437 mainly belonged to both cellular and metabolic process in GO biological process gene sets.

438

#### 439 *Gene set enrichment analysis in deconvoluted ICM cells' genes expression according to* 440 *maternal age*

441 After Entrez Gene ID conversion, 12,344 genes were considered expressed in ICM. In the GO Biological  
442 Process database, 106 identified gene sets differed according to maternal age in the ICM (11 enriched

443 in OM and 95 enriched in YM) (Additional file 7). In the KEGG database, 14 gene sets were perturbed  
444 (Additional file 7).

445 Using SUMER analysis (Figure 5), gene sets enriched in OM ICM were mainly grouped under terms  
446 “Respiratory electron transport chain” and “Translational elongation”. The first term represents  
447 mitochondrial function while the second represents translation function. Moreover, terms relative to  
448 “Systemic lupus erythematosus” are conserved by SUMER analysis and enriched in OM ICM. Genes  
449 involved in these enrichments were also present in dysregulated metabolic pathways.

450 Gene sets enriched in YM ICM were involved in different biological processes such as “Mitotic nuclear  
451 division” which is relative to mitosis. Under this term, “Spindle organization” and “Negative regulation  
452 of supramolecular fiber organization” terms represent the assembly of spindle during mitosis.  
453 “Chromosome segregation” is also found in this grouping. Furthermore, terms under “Golgi vesicle  
454 transport” seem to be involved in cytokinesis and vesicle trafficking. The catabolism of protein and  
455 mRNA is represented by “Ubiquitin mediated proteolysis”. Translation function is represented by  
456 several terms grouped under “covalent chromatin modification” in the mitosis part.

457 “Leukocyte transendothelial migration” term is also enriched in YM ICM and seems to represent cell  
458 signaling and adhesion. Indeed, this term regroups several pathways such as “Focal adhesion”,  
459 “Adherens junction” and “WNT signaling pathway”.

460 Finally, further terms related to embryo development could be found in different groups such as  
461 “Dendrite development”, “Cell differentiation in spinal cord” or “endothelial cell development”.

462

#### 463 *Gene set enrichment analysis in TE*

464 After Entrez Gene ID conversion, 11,874 genes were considered expressed in TE from OM or YM  
465 embryos. In GO BP database, 38 gene sets were perturbed by mares’ age in the TE of embryos (11  
466 enriched in YM and 27 enriched in OM) (Additional file 8). In the KEGG database, 5 pathways were  
467 enriched in the TE of OM embryos (Additional file 8).

468 As in ICM part, using SUMER analysis, translation (represented by both “Translational termination” and  
469 “Ribosome” terms) and mitochondrial function (represented by “Oxidative phosphorylation”  
470 associated terms) were enriched in OM embryos. “Proteasome” term is enriched in OM embryos  
471 representing protein catabolism.  
472 Moreover, transcription (represented by “Peptidyl lysine trimethylation” term) and embryo  
473 development (represented by “spinal cord development”) were, as in ICM, also, enriched in YM  
474 embryos. Finally, in TE, “Regulation of action potential” term and associated terms were enriched in  
475 YM. These terms could be related to ion movement.

476

## 477 **Discussion**

478 Maternal aging did not affect embryo recovery rate, embryo size nor total RNA quantity. Moreover,  
479 total RNA content in equine embryo was exponentially linked to embryo diameter and was not affected  
480 by maternal age.

481 As in tumor, today, the only way to analyze separate expression of ICM and TE cells in mammalian  
482 embryos is to use micromanipulation but micromanipulation is expensive in time and requires adapted  
483 skills and material. In this study, micromanipulation to properly separate ICM and TE was not possible.  
484 Therefore, deconvolution appears to be a good option to analyze the effects of maternal ICM gene  
485 expression in mixed ICM and TE samples. Deconvolution seemed to have been effective on embryo to  
486 separate ICM and TE gene expression. Indeed, the 341 genes differentially expressed in ICMandTE vs  
487 TE\_part were also identified by comparing TE with post-deconvolution ICM cells gene expression  
488 dataset. Moreover, after deconvolution, more genes were differentially expressed between TE and  
489 ICM datasets and among them, more genes identified in literature were differentially expressed.  
490 Finally, PCA showed that deconvolution separate DeMixT\_ICM\_cells from other datasets while before  
491 deconvolution ICMandTE and TE\_part were partly superposed. Only POU5F1 and UTF1 differed in  
492 terms of allocation between previously published data [23] and the present study. In data before  
493 deconvolution, expression of POU5F1 and UTF1 were extremely variable according to embryo

494 diameter in both compartments. Deconvolution reduced the variability of both gene expression in ICM  
495 but not in TE. Studies showed that POU5F1 is expressed in both compartments until Day 10 post  
496 ovulation in equine in vivo produced embryos [47] but its expression decreases progressively from Day  
497 7 in TE cells. Thus, the statistically increased expression of POU5F1 in TE on Day 8 is compatible with  
498 previously published data.

499 Therefore, it was chosen to only present the analysis of the effects of age in DeMixT\_ICM\_cells dataset.  
500 In all cases, deconvolution did not change the principal results of maternal aging on embryo gene  
501 expression because the comparison of maternal age in ICMandTE and DeMixT\_ICM\_cells gave  
502 comparable results.

503 In both ICM and TE, the expression of genes involved in mitochondrial function and translation with  
504 ribosome biogenesis were enriched in OM embryos while transcription with chromatin modification  
505 was enriched in YM embryos (Figure 6). Mitosis, signaling and adhesion pathways were additionally  
506 altered and the expression of genes involved in embryo development was particularly reduced in the  
507 ICM hemi-embryos from old mares. Finally, in the TE, ion movement related genes were affected.

508 Several studies on mature oocytes in women and mice, using microarrays and RNAseq, had shown a  
509 differential expression of genes involved in mitochondrial, cell cycle, cytoskeleton functions and  
510 related pathways according to maternal age [48–50], as observed in the present study in equine  
511 blastocysts, especially in the ICM. In equine follicles and oocytes, gene expression, as studied by RT-  
512 qPCR, quantitatively and temporally differed with maternal age, suggesting a desynchronization of  
513 essential genes for maturation [51]. Moreover, a study on the expression of 48 genes in cumulus-  
514 oocytes complexes from young (< 12 years of age) and old (> 18 years old) mares showed differential  
515 expression that may be related with the reduced developmental competence observed in old mares'  
516 oocytes [52]. Finally, genes related to mitochondrial replication and function were down-regulated in  
517 oocytes of old mares [16]. Even effects of maternal aging on gene expression in equine oocytes, using  
518 high throughput techniques, are not yet available, results on equine Day 8 blastocysts suggest that  
519 mitochondrial perturbations present in the oocyte persist until the blastocyst stage. In humans, single-

520 embryo RNA-seq analysis showed that maternal aging alters the transcriptome of blastocysts derived  
521 from ICSI. Genes altered by maternal age were related to segregation of chromosomes, embryonic  
522 growth and cell signaling [53,54], as observed in our study on equine blastocysts.

523 The equine embryo remains spherical, grows very fast and is surrounded by its glycoprotein capsule  
524 up to 22 days post ovulation [55]. At 8 days post ovulation, equine embryos are therefore growing and  
525 the endodermal layer entirely delineates the future yolk sac (Figure 6) [56]. Moreover, whereas in Day  
526 7 embryos, the epiblast is lined by a continuous layer of trophoblast named the polar trophoblast. As  
527 the blastocyst grows, epiblast cells phagocyte polar trophoblast cells that become discontinuous.  
528 During this time, epiblast and polar trophoblast cells divided and are bound by tight junctions [57]. The  
529 turnover in focal adhesion is essential for cell movement and phagocytosis [58].

530 Cell signaling and adhesion function were altered in OM embryos. Indeed, KEGG pathways "focal  
531 adhesion" and "adherens junction" regrouped in "Leukocyte transendothelial migration" on Figure 4  
532 are disturbed in old mares' embryos. Focal adhesions are subcellular structures observed in adherent  
533 cells, that are clusters of different structural and signaling molecules and integrins that form a physical  
534 link between the actin cytoskeleton and the extracellular matrix [59,60]. Integrins are the major  
535 receptors for cell adherence to extracellular matrix proteins in focal adhesions. Indeed, they play an  
536 important role in transmembrane connections to the cytoskeleton and activation of several signaling  
537 pathways, as growth factor receptors [61]. *Integrin Subunit Alpha V (ITGAV)* expression was reduced  
538 in the ICM ( $\log_2FC = -1.66$ ;  $FDR < 0.05$ ) of embryos collected from OM. In different human breast cancer  
539 cell lines, cell proliferation, invasion and renewal are restricted when *ITGAV* is inhibited [62].  
540 Therefore, the reduction of *ITGAV* could lead to an impaired cell signalization in OM embryos.

541 Moreover, small GTPases within the Rho family are required for the turnover of actin filaments and  
542 cell adhesions necessary for cell migration, that are essential processes for the proper development of  
543 the embryo. Activity and localization of each Rho GTPase must be precisely regulated as the  
544 communication between them is indispensable to achieve this process [63]. The *Rho GTPase Activating*  
545 *Protein 9* and *35 (ARHGAP9 and ARHGAP35)* were downregulated (respectively,  $\log_2FC = -1.83$ ,  $FDR <$

546 0.05 and  $\log_2FC = -0.74$ ,  $FDR < 0.05$ ) in OM ICM. *ARHGAP35* codes for P190 Rho GTPase activating  
547 protein that mediates signalization through integrin-dependent adhesion in cells. Aberrant neural  
548 morphogenesis, related to an accumulation of polymerized actin in embryoneural tube cells, is  
549 reported in mice lacking P190 Rho GTPase activating proteins [64]. Furthermore, P21 activated kinases  
550 (PAK) are effector kinases for other small Rho GTPases such as Rac and regulate the polymerization of  
551 actin and can affect the microtubule organization and therefore the regulation of cytoskeleton [65].  
552 Once activated by this signaling pathway, PAKs promote the turnover of focal adhesions. PAKs will  
553 then, trigger p38 complex activation that regulates transcription [66]. *PAK3* was downregulated in the  
554 OM ICM ( $\log_2FC = -3.76$ ,  $FDR < 0.001$ ). However, in TE, *SLIT-ROBO Rho GTPase Activating Protein 3*,  
555 *SRGAP3* was one of the upregulated genes in OM ( $\log_2FC = 2.52$ ,  $FDR < 0.01$ ). This gene regulates  
556 cytoskeletal reorganization and plays an important role in the formation of cell-cell adhesion [67].  
557 Therefore, maternal aging seems to alter focal adhesion in both ICM and TE of embryos but, while in  
558 ICM, it seems to reduce cohesion and signalization, in TE it seems to reinforce them.  
559 Several signaling pathways required for development were altered and numerous genes related to  
560 focal adhesions and signaling pathways were down-regulated in OM ICM (*ARHGAP9* and *ARHGAP35*;  
561 *B-Raf Proto-Oncogene, Serine/Threonine Kinase, BRAF*; *Filamin C, FLNC*; *FYN Proto-Oncogene, Src*  
562 *Family Tyrosine Kinase, FYN*; *PAK3*; *Protein Phosphatase 1 Regulatory Subunit 12A, PPP1R12A*; *Talin 2*,  
563 *TLN2*; *X-Linked Inhibitor Of Apoptosis, XIAP*). Among all signaling pathways, transforming growth factor  
564 beta (TGF- $\beta$ ), Wg et Int (Wnt), mitogen-activated protein kinases (MAPK) and apoptosis signaling were  
565 altered by maternal age in equine embryos. Indeed, *transforming growth factor beta receptor 1*  
566 (*TGFBR1*) was downregulated ( $\log_2FC = -4.34$ ;  $FDR < 0.05$ ) in the ICM of embryos from old mares.  
567 *TGFBR1* is one of the two dimers for the receptor for transforming growth factors beta. This factor  
568 family is essential in the generation of axes and cell fate commitment during mammal embryogenesis  
569 [68]. Therefore, the reduced production of TGF- $\beta$  receptor in ICM could impair the establishment of  
570 cell lines in old mares' embryos. Moreover, *Casein Kinase 1 Alpha 1, (CSNK1A1)* and *Protein*  
571 *Phosphatase 2 Regulatory Subunit B'Epsilon (PPP2R5E)*, involved in Wnt signaling pathway, were

572 downregulated in OM embryos. Wnt signaling pathway is also involved in nervous development  
573 system and may act on cell determination in mice embryos [69]. In horses, gastrulation begins by Day  
574 12 post ovulation and the primitive streak develops quickly after mesoderm formation [70]. It is  
575 therefore possible that the alteration of the Wnt signaling pathway could have a negative impact on  
576 the brain development of the future fetus. This hypothesis could be confirmed by the fact that *DMRT3*  
577 (*Doublesex And Mab-3 Related Transcription Factor 3*) was downregulated in the OM ICM (log<sub>2</sub>FC = -  
578 3.07, FDR < 0.05). *DMRT3* is required for the proper development of the brain in prenatal chicken and  
579 mice [71]. Moreover, mutations of this gene are responsible for different gaits in horses (specific to  
580 aptitude to trot instead of cantering) [72]. Therefore, altogether, the data indicate that cell fate  
581 commitment in ICM seems to be altered by maternal aging.

582 Cell cycle related pathways and genes are also disturbed by maternal aging. Indeed, the expression of  
583 *Pleckstrin Homology domain-Interacting Protein (PHIP)*, also known as replication initiation  
584 determinant, was reduced (log<sub>2</sub>FC = -0.86, FDR < 0.01) in the OM ICM. This gene is required for the  
585 initiation of DNA replication. Fibroblasts originated from mice embryos with depleted *PHIP* expression  
586 presented less replication-initiation as well as abnormal replication fork progression events [73].  
587 Moreover, several cyclins (CCN) were less expressed in the ICM of embryos from old mares (*CCNB3*,  
588 *CCNI* and *CCNT2*). Cyclins are key cell cycle regulators and, for instance cyclin B3 degradation allows  
589 the passage from metaphase (chromatids aligned on metaphase plate) to anaphase (segregation of  
590 sister chromatids) [74]. During metaphase and anaphase, spindle assembly and chromosome  
591 segregation could also be compromised. Indeed, genes involved in the formation of microtubules  
592 (*Tubulin Gamma 2, TUBG2*) [75], the generation of the mitotic spindle (*Never in Mitosis A related kinase*  
593 *9, NEK9*) [76], the production of cohesion and condensing complexes (*Structural Maintenance Of*  
594 *Chromosomes 4, SMC4*) [77], the control of centriole length, important for centrosomes and  
595 microtubules organization (*Centriolar Coiled-Coil Protein 110, CCP110*) [78], the amplification of intra-  
596 spindle microtubules allowing the formation of a sufficient quantity of kinetochore (*HAUS Augmin Like*  
597 *Complex Subunit 6, HAUS6*) [79], the stabilization and protection of the cohesin complex association

598 with chromatin (*Cell Division Cycle Associated 5, CDCA5; shugosin 2, SGO2*) [80,81] and the production  
599 of microtubule-based motor proteins (*Kinesin Family Member 14 and 23, KIF14 and KIF23*) [82] were  
600 downregulated in OM ICM. Furthermore, *Centromere protein F (CENPF, log2FC = -1.11, FDR < 0.0001)*,  
601 an important regulator of chromosome segregation during mitosis, was reduced in OM ICM. CENPF is  
602 indispensable for murine embryo development from the first stages of development until at least the  
603 blastocyst stage [83]. Impaired CENPF expression could induce chromosome mis-segregation in the  
604 ICM of old mares' embryos.

605 Cytokinesis seems to also be altered by maternal age. *CD2-associated protein (CD2AP)* and *Vacuolar*  
606 *Protein Sorting 4 Homolog A (VPS4A)* were downregulated in OM ICM (respectively, log2FC = -0.90,  
607 FDR < 0.05 and log2FC = -0.77, FDR < 0.05). Both are involved in the late phase of cytokinesis which  
608 correspond to the abscission of the cell, producing two daughter cells [84,85]. Globally, results suggest  
609 that cell division is impaired or indicate that there is less division in the ICM of old mares' embryos.

610 Transcription is regulated by epigenetic marks. Several pathways linked to methylation and acetylation  
611 were enriched in YM ICM and TE in relation to numerous upregulated genes in YM ICM (i.e., *Histone*  
612 *Deacetylase 1 and 2, HDAC1 and HDAC2; Helicase, Lymphoid Specific, HELLS; DNA Methyltransferase 3*  
613 *Alpha, DNMT3A; Lysine Acetyltransferase 6B, KAT6B; Lysine Demethylase 4A, KDM4A; Lysine*  
614 *Methyltransferase 2A, KMT2A; Nuclear Receptor Binding SET Domain Protein 3, NSD3; SET Domain*  
615 *Containing 5, SETD5; Tet Methylcytosine Dioxygenase 1, TET1*). At blastocyst stage, *de novo*  
616 methylation is more important in the inner cell mass than in trophoblast cells and is essential for the  
617 proper development of mammalian embryos [86]. *DNMT3A* is essential for *de novo* methylation and  
618 required for embryo viability [87]. *DNMT3A* expression was reduced in OM ICM (log2FC = -1.01, FDR <  
619 0.05). As it has been shown that maternal aging increases the acetylation of lysine 12 of histone H4 in  
620 mice oocytes, affecting fertilization and embryo development [88], it is likely that the methylation and  
621 acetylation dysregulation observed in the embryos of old mares could be inherited from the oocyte  
622 rather than directly due to embryo environment.

623 Many of the perturbed gene sets were involved in protein metabolism. In OM ICM, translation was  
624 enriched but protein catabolism is not, whilst in TE, both are enriched. The biogenesis of ribosomes  
625 and protein metabolism regulation are crucial for embryo development. Still in the ICM, proteasome  
626 and mRNA catabolism pathways were also affected. In embryonic stem cells, the proteasome plays a  
627 role in the restriction of permissive transcriptional activity by promoting a dynamic turnover of  
628 transcription factors [89].

629 Both in OM ICM and TE, several pathways related to mitochondrial function were enriched.  
630 Mitochondria are long known to be the “powerhouse” of cells, producing most of the necessary ATP  
631 for cellular function [90]. ATP production is possible through oxidative phosphorylation enabled by the  
632 electron transport chain in the inner membrane of mitochondria. This process begins with the  
633 oxidation of NADH or FADH that generates a proton gradient across the mitochondrial membrane,  
634 driving ATP synthesis through the phosphorylation of ADP *via* ATPase [91]. Enriched pathways in OM  
635 embryos demonstrate that the NADH dehydrogenase (complex I), the oxidoreductase acting on a  
636 heme group of donors (complex III) with the global electron carrier, and the proton-transporting ATP  
637 synthase (1<sup>st</sup> part of the complex V) activities were particularly enriched while the second part of  
638 complex V is depleted. Since all complexes are required to produce ATP, the impaired complex  
639 generation may lead to mitochondrial stress and uncoupled respiratory chain, resulting in poor ATP  
640 production. In human IVF embryos, reduced mitochondrial respiration efficiency, with a negative effect  
641 on embryo development, is observed when oocytes are collected from old women [92]. In mammalian  
642 expanded blastocysts, energy requirement is high, probably because of the need for ion transport  
643 enabling quick blastocoel cavity expansion. Defects in metabolic requirements during blastocyst  
644 development are associated with impaired implantation [93]. Equine embryos are not an exception  
645 [94].

646 Mitochondria being maternally inherited, uncoupling of the mitochondrial respiratory chain may  
647 originate from mitochondrial defects already present in oocytes [95]. Studies have shown that, in  
648 human, poor oocyte quality is responsible for the decline in fertility associated with maternal aging.

649 The reduced oocyte developmental competence in older women is related to mitochondrial  
650 dysfunction [96]. Regardless of the cell type, it is well known that mitochondrial DNA is more sensitive  
651 to mutation than nuclear DNA and that aging is an important risk factor. In mammalian oocytes,  
652 mitochondrial DNA mutations increase with age and are inversely correlated with oocyte quality [97–  
653 99]. In mice, oocyte mitochondrial DNA content increases during *in vivo* oogenesis without difference  
654 according to mice age [100]. In horses' oocytes, before *in vitro* maturation, no difference between  
655 young and old mares is observed while after, reduced mitochondrial DNA copy numbers, increased  
656 swelling and reduced mitochondrial cristae are reported in oocytes collected from mares >12 years.  
657 This suggests that oocyte mitochondria from old mares present deficiencies that become problematic  
658 when energy needs are high, as during *in vitro* maturation or subsequent embryo development [101].  
659 Because mitochondrial replication in equine embryos does not begin until the blastocyst stage [102],  
660 oocyte mitochondrial numerical or functional deficiencies may compromise early embryogenesis.  
661 Alternatively, perturbations in mitochondrial gene expression as observed in old mares' embryos may  
662 also result from alterations of the maternal environment. Indeed, mitochondrial function can be  
663 improved in mouse embryos by adding antioxidants to the *in vitro* culture medium [103]. To our  
664 knowledge, there is no study on oviductal or uterine fluid composition according to maternal age at  
665 least in the horse, at the present time.

666 Finally, ATP production defects could affect embryo blastocoel expansion. Indeed, in TE, the GO  
667 biological process "Regulation of action potential" and "Transmission nerve impulse", related to ion  
668 movement, were enriched in YM embryos. These processes are associated with genes involved in the  
669 action potential dependent ion channels. These ion channels such as  $Na^+$ ,  $K^+$ , ATPase are essential for  
670 the accumulation of blastocoel and subsequently yolk sac fluid in equine embryos [104]. Their  
671 inhibition is detrimental for embryo development [105]. Thus, both reduced ATP production and  
672 disturbed ion channels' function may impact embryo growth. Studies on blastocyst growth and size  
673 according to mares' age, however, are controversial [9–15], probably due to the fact that the size of  
674 the embryo at the same stage is very variable, as it is the case in this study. To this time, no study on

675 the effect of mares' age on equine  $NA^+$ ,  $K^+$ , ATPase abundance and function in equine embryos is  
676 available.

677 Altogether these data indicate that several processes related to chromatin segregation, cytokinesis,  
678 apposition of epigenetic marks and mitochondrial function are affected in older mares' embryos and  
679 that the ICM appears to be more affected than the TE. These perturbations most certainly contribute  
680 to the increased subfertility and early embryo loss observed in aged mares [2,6,7]

681

## 682 **Conclusion**

683 This is the first study showing an effect of maternal age on gene expression in the equine blastocyst at  
684 Day 8 post ovulation. Maternal age, even for mares as young as 10 years old, disturbs mitosis,  
685 translation and transcription, cell signaling and adhesion as well as mitochondrial function and cell  
686 commitment in horse embryos. These perturbations on Day 8 post ovulation may affect the further  
687 development of the embryo and as a result, may contribute to decreased fertility due to aging. Even if  
688 the oocyte seems to be in great part responsible for these alterations, maternal environment could  
689 also be responsible. So far, maternal age does not appear to have an impact on foal morphology at  
690 birth but, as for placental function, more subtle effects may exist. Here, as embryos were collected  
691 long before implantation, it is impossible to know if these embryos would have implanted and if they  
692 would have produced a live foal or not. Studies on long-term growth and health of foals born to the  
693 same mares are currently being performed.

694

## 695 **List of abbreviations**

696 ARHGAP35: Rho GTPase Activating Protein 35

697 ARHGAP9: Rho GTPase Activating Protein 9

698 BRAF: B-Raf Proto-Oncogene, Serine/Threonine Kinase

- 699 CCN: cyclin
- 700 CCP110: Centriolar Coiled-Coil Protein 110
- 701 CD2AP: CD2-associated protein
- 702 CD69: CD69 Molecule
- 703 CDCA5: Cell Division Cycle Associated 5
- 704 CENPF: Centromere protein F
- 705 CSNK1A1: Casein Kinase 1 Alpha 1
- 706 DEG: differential expressed genes
- 707 DeMixT\_ICM\_cells: deconvoluted gene expression in ICM cells
- 708 DeMixT\_TE\_cells: deconvoluted gene expression in TE cells
- 709 DMRT3: Doublesex And Mab-3 Related Transcription Factor 3
- 710 DNMT3A: DNA Methyltransferase 3 Alpha
- 711 ERR: embryo collection rate
- 712 FDR: false discovery rate
- 713 FLNC: Filamin C
- 714 FYN: FYN Proto-Oncogene, Src Family Tyrosine Kinase
- 715 GO BP: Gene Ontology biological process
- 716 GO: Gene Ontology
- 717 GSEA: gene set enrichment analyses
- 718 HAUS6: HAUS Augmin Like Complex Subunit 6

- 719 HDAC: Histone Deacetylase
- 720 HELLS: Helicase, Lymphoid Specific
- 721 ICM: inner cell mass
- 722 ICMandTE: inner cell mass enriched hemi-embryo
- 723 ICSI: intracytoplasmic sperm injection
- 724 ITGAV: Integrin Subunit Alpha V
- 725 KAT6B: Lysine Acetyltransferase 6B
- 726 KDM4A: Lysine Demethylase 4A
- 727 KEGG: Kyoto
- 728 KEGG: Kyoto Encyclopedia of Genes and Genomes
- 729 KIF: Kinesin Family Member
- 730 KMT2A: Lysine Methyltransferase 2A
- 731 Log2FC: log2 fold change
- 732 MAPK: mitogen-activated protein kinases
- 733 NEK9: Never in Mitosis A related kinase 9
- 734 NES: normalized enrichment score
- 735 NSD3: Nuclear Receptor Binding SET Domain Protein 3
- 736 OM: old mares
- 737 PAK: P21 activated kinases
- 738 PHIP: Pleckstrin Homology domain-Interacting Protein

- 739 PLAC8A: placenta Associated 8 A
- 740 POU5F1: POU Class 5 Homeobox 1
- 741 PPP1R12A: Protein Phosphatase 1 Regulatory Subunit 12A
- 742 PPP2R5E: Protein Phosphatase 2 Regulatory Subunit B'Epsilon
- 743 ROCK: Rho-associated coiled-coil kinases
- 744 RPS29: Ribosomal Protein S29
- 745 SETD5: SET Domain Containing 5
- 746 SGO2: shugosin 2
- 747 SMC4: Structural Maintenance Of Chromosomes 4
- 748 SRGAP3: SLIT-ROBO Rho GTPase Activating Protein 3
- 749 SRY: sex determining region Y
- 750 TE: trophoblast
- 751 TE\_part: pure trophoblast hemi-embryo
- 752 TET1: Tet Methylcytosine Dioxygenase 1
- 753 TGF-  $\beta$ : transforming growth factor beta
- 754 TGFBR1: transforming growth factor beta receptor 1
- 755 TLN2: Talin 2
- 756 TUBG2: Tubulin Gamma 2
- 757 UTF1: Undifferentiated Embryonic Cell Transcription Factor 1
- 758 VPS4A: Vacuolar Protein Sorting 4 Homolog A

759 Wnt: Wg et Int

760 XIAP: X-Linked Inhibitor Of Apoptosis

761 XIST: X inactive Specific Transcript

762 YM: young mares

763

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1052

## 1053 **Figures**

1054 Figure 1: Bisection of equine embryos at 8 days post-ovulation into an ICMandTE and a TE\_part.

1055 A) Step 1: Identification of the ICM and the TE; B) Step 2: Definition of the cutting plane to isolate the  
1056 ICM in one of the parts; C) Step 3: Cutting of the embryo and separation of the two parts. ICMandTE:  
1057 inner cell mass + trophoblast; TE part: pure trophoblast.

1058 NB: Here the trophoblast represents trophectoderm + endoderm and ICM is composed of epiblast  
1059 cells.

1060

1061 Figure 2: Total RNA yield per embryo in relation to embryo diameter

1062 Green circles represent embryos from young mares (YM) and yellow squares represent embryos from  
1063 old mares (OM). The used relation is exponential.

1064

1065 Figure 3: Gene expression in ICM and TE before and after deconvolution using DeMixT

1066 A) Venn diagrams of the differential gene expression in ICMandTE vs TE part (before deconvolution),  
1067 DeMixT\_ICM\_cells vs DeMixT\_TE\_cells (after deconvolution) and DeMixT\_ICM\_cells vs TE\_part (gene  
1068 expression of ICM after deconvolution vs gene expression in TE\_part without deconvolution); B)  
1069 Principal Component Analysis of gene expression of DeMixT\_ICM\_cells, DeMixT\_TE\_cells, ICMandTE  
1070 and TE part datasets.

1071 Deconvolution was used to isolate gene expression of ICM and TE cells in ICMandTE hemi-embryos.  
1072 ICMandTE: inner cell mass + trophoblast; TE part: pure trophoblast. Here trophoblast represents  
1073 trophectoderm + endoderm.

1074

1075 Figure 4: Analysis of differentially expressed genes (DEG) in embryos according to maternal age

1076 A) representation of down (blue) and upregulated (red) DEG in ICM (from DeMixT\_ICM\_cells data  
1077 obtained after deconvolution of ICMandTE using DeMixT R package [33,34]) and TE (from TE\_part  
1078 dataset) of embryos from OM vs YM. Functional classifications (for down regulated genes in ICM and  
1079 TE and for up-regulated genes in ICM) using GO Biological Process obtained with PANTHER online  
1080 software are presented as pie charts. For upregulated DEG in TE, no functional classification was made.

1081 B) Bar chart presenting p-adjusted of most specific subclass pathways provided by PANTHER online  
1082 software after statistical overrepresentation test (Fisher's Exact test and Benjamini-Hochberg  
1083 correction) with the Human GO Biological Process annotation on down-regulated DEGs in ICM.

1084 DEG: Differentially Expressed Genes (FDR < 0.05); TE: Trophoblast; ICM: Inner Cell Mass; OM: Old  
1085 multiparous mares; YM: young multiparous mares

1086

1087 Figure 5: GSEA clustering of the most perturbed terms in ICM and TE according to mares' age

1088 Nodes represent altered gene sets in ICM and TE (FDR < 0.05). Node size represents the gene set size.

1089 Node shape represents the gene set database: GO BP (circle) or KEGG (diamond). Blue nodes represent

1090 enriched gene sets in YM (NES < 0) while red nodes represent enriched gene sets in OM (NES > 0).

1091 Edges represent the level of connection between representative gene sets. This graph was performed

1092 using SUMER R package [44] and modified using cytoscape 3.8.2 [45]

1093 ICM: Inner Cell Mass; TE: trophoblast; FDR: False Discovery Rate; GO BP: Gene Ontology Biological

1094 Process; Kyoto Encyclopedia of Genes and Genomes; NES: Normalized Enrichment Score; YM: Young

1095 mares; OM: Old mares

1096

1097 Figure 6: Schematic representation of the observed effects of maternal aging on ICM and TE in equine

1098 blastocysts

1099 Normal blastocyst development from day 7 to day 9 is represented inside black square using literature

1100 [56,57]. Light blue boxes represent biological processes that are enriched in YM embryos and light red

1101 boxes represent biological processes that are enriched in OM embryos.

1102

1103 Additional file 1:

1104 Additional file 1.png

1105 Pipeline of biostatistical analysis

1106 Once extracted, total RNA qualification and quantification were obtained using a Bioanalyzer. For  
1107 sequencing, 5 ng/sample of total RNA were used for paired end RNA sequencing (I2BC platform) using  
1108 Illumina NextSeq 500 high technology. Sequences were trimmed using Cutadapt. To determine XIST  
1109 sequence, a *de novo* assembly was made using a mapping with STAR and StringTie. The GTF observed  
1110 was aligned in IGV and XIST sequence was found at the known position (according to Pablo Ross  
1111 personal communication). XIST sequence was included in EquCab 3.0 and a new mapping was  
1112 performed using STAR. Counting was performed using featureCounts. The differential analysis was  
1113 performed including embryo sex and diameter using Deseq2. A functional enrichment analysis of DEGs  
1114 was performed using PANTHER web software. In another time, counts of all genes were normalized  
1115 using Deseq2 and a gene set analysis (GSEA) was performed using the Gene Ontology (GO) biological  
1116 process (BP) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases with GSEA software  
1117 from the Broad Institute. To reduce redundancy between terms, SUMER analysis was performed on  
1118 enriched gene sets.

1119

1120 Additional file 2:

1121 Additional file 2.pdf

1122 Analysis of ICM enriched and TE part before gene expression deconvolution using DeMixT

1123 On first page, the analysis of differential expressed genes is represented. The second page represent  
1124 the results of the gene set enrichment analysis of ICMandTE gene expression. The third page presents  
1125 the clustering of gene sets altered by maternal age from SUMER analysis of the GO BP and KEGG terms  
1126 of mixed ICMandTE and TE\_part.

1127

- 1128 Additional file 3:
- 1129 Additional file 3.csv
- 1130 Differential gene analysis using DeSeq2 in ICMandTE of equine embryo at Day 8 post-ovulation
- 1131 Equine ensemble ID, orthologue human Ensembl ID, Orthologue human Entrez Gene ID, gene  
1132 description, normalized counts for each sample of ICM and parameters obtained after Deseq2 analysis  
1133 (log2FoldChange, pvalue and padj (after FDR correction)) of genes expressed in ICM enriched part  
1134 (before gene expression deconvolution using DeMixT) of OM and YM embryos
- 1135 ICM: Inner cell mass; OM: old mares; YM: young mares
- 1136
- 1137 Additional file 4:
- 1138 Additional file 4.csv
- 1139 Gene set enrichment analysis results on gene expression comparing ICMandTE of embryos from old  
1140 mares to young mares
- 1141 Gene Set Enrichment Analysis results (pathway name, GO accession when possible and size,  
1142 Normalized Enrichment Score, p-value and FDR corrected q-value) for GO biological process and KEGG  
1143 databases on ICMandTE gene expression table.
- 1144
- 1145
- 1146 Additional file 5:
- 1147 Additional file 5.csv
- 1148 Differential gene analysis using DeSeq2 in DeMixT\_ICM\_cells of equine embryo at Day 8 post-ovulation

1149 Equine ensemble ID, orthologue human Ensembl ID, Orthologue human Entrez Gene ID, gene  
1150 description, normalized counts for each sample of ICM and parameters obtained after Deseq2 analysis  
1151 (log2FoldChange, pvalue and padj (after FDR correction)) of genes expressed in ICM (after gene  
1152 expression deconvolution of ICMandTE using DeMixT) of OM and YM embryos

1153 ICM: Inner cell mass; OM: old mares; YM: young mares

1154

1155 Additional file 6:

1156 Additional file 6.csv

1157 Differential gene analysis using DeSeq2 in TE\_part of equine embryo at Day 8 post-ovulation

1158 Equine ensemble ID, orthologue human Ensembl ID, Orthologue human Entrez Gene ID, gene  
1159 description, normalized counts for each sample of ICM and parameters obtained after Deseq2 analysis  
1160 (log2FoldChange, pvalue and padj (after FDR correction)) of genes expressed in TE of OM and YM  
1161 embryos

1162 TE: Trophoblast; OM: old mares; YM: young mares

1163

1164 Additional file 7:

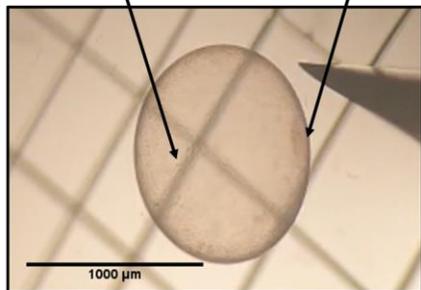
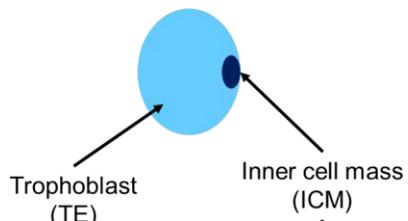
1165 Additional file 7.csv

1166 Gene set enrichment analysis results on gene expression of DeMixT\_ICM\_cells of embryos from young  
1167 and old mares

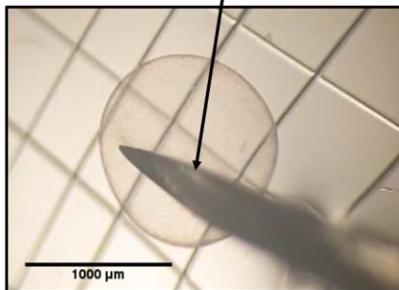
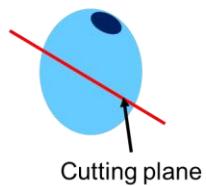
1168 Gene Set Enrichment Analysis results (pathway name, GO accession when possible and size,  
1169 Normalized Enrichment Score, p-value and FDR corrected q-value) for GO biological process and KEGG

1170 databases on DeMixT\_ICM\_cells gene expression table (after gene expression deconvolution on  
1171 ICMandTE using DeMixT).  
  
1172  
  
1173 Additional file 8:  
  
1174 Additional file 8.csv  
  
1175 Gene set enrichment analysis results on gene expression of TE\_part of embryos from young and old  
1176 mares  
  
1177 Gene Set Enrichment Analysis results (pathway name, GO accession when possible and size,  
1178 Normalized Enrichment Score, p-value and FDR corrected q-value) for GO biological process and KEGG  
1179 databases on TE\_part gene expression table.

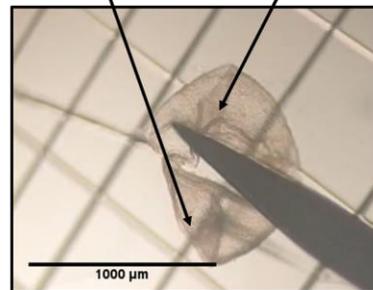
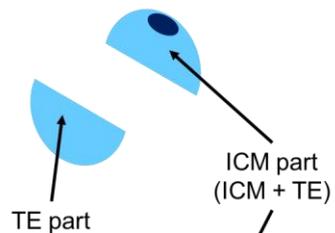
A)

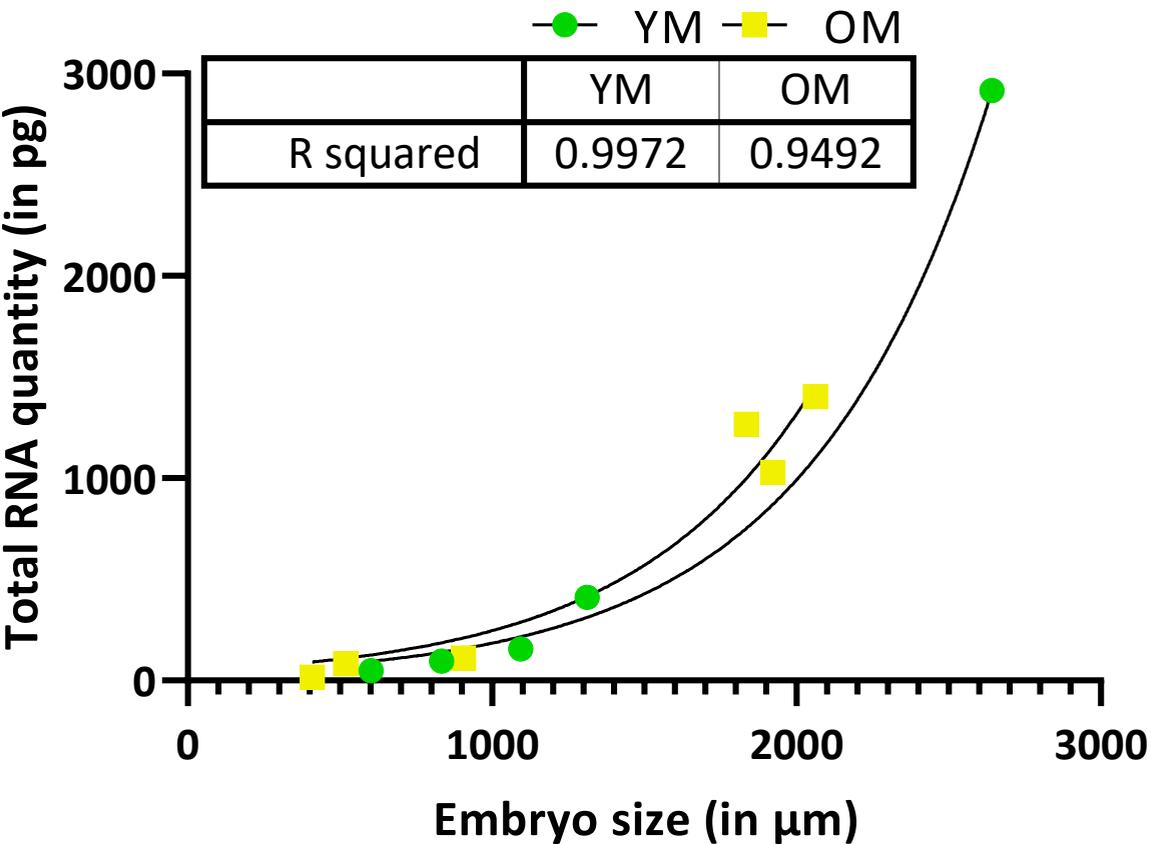


B)



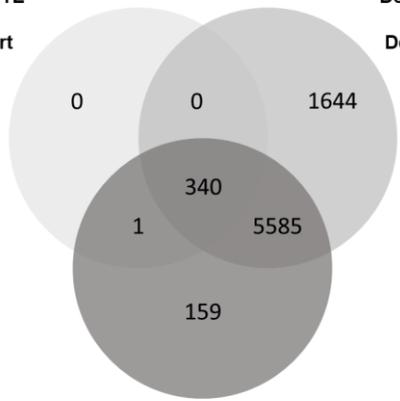
C)





**A**

ICMandTE  
vs  
TE\_part

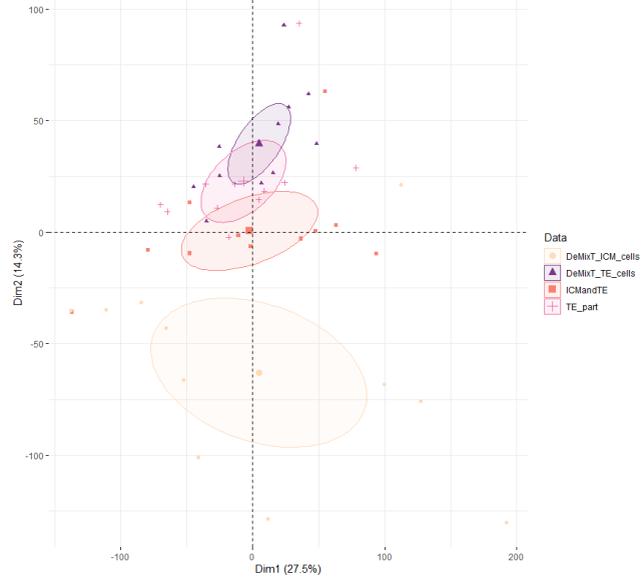


DeMixT\_ICM\_cells  
vs  
DeMixT\_TE\_cells

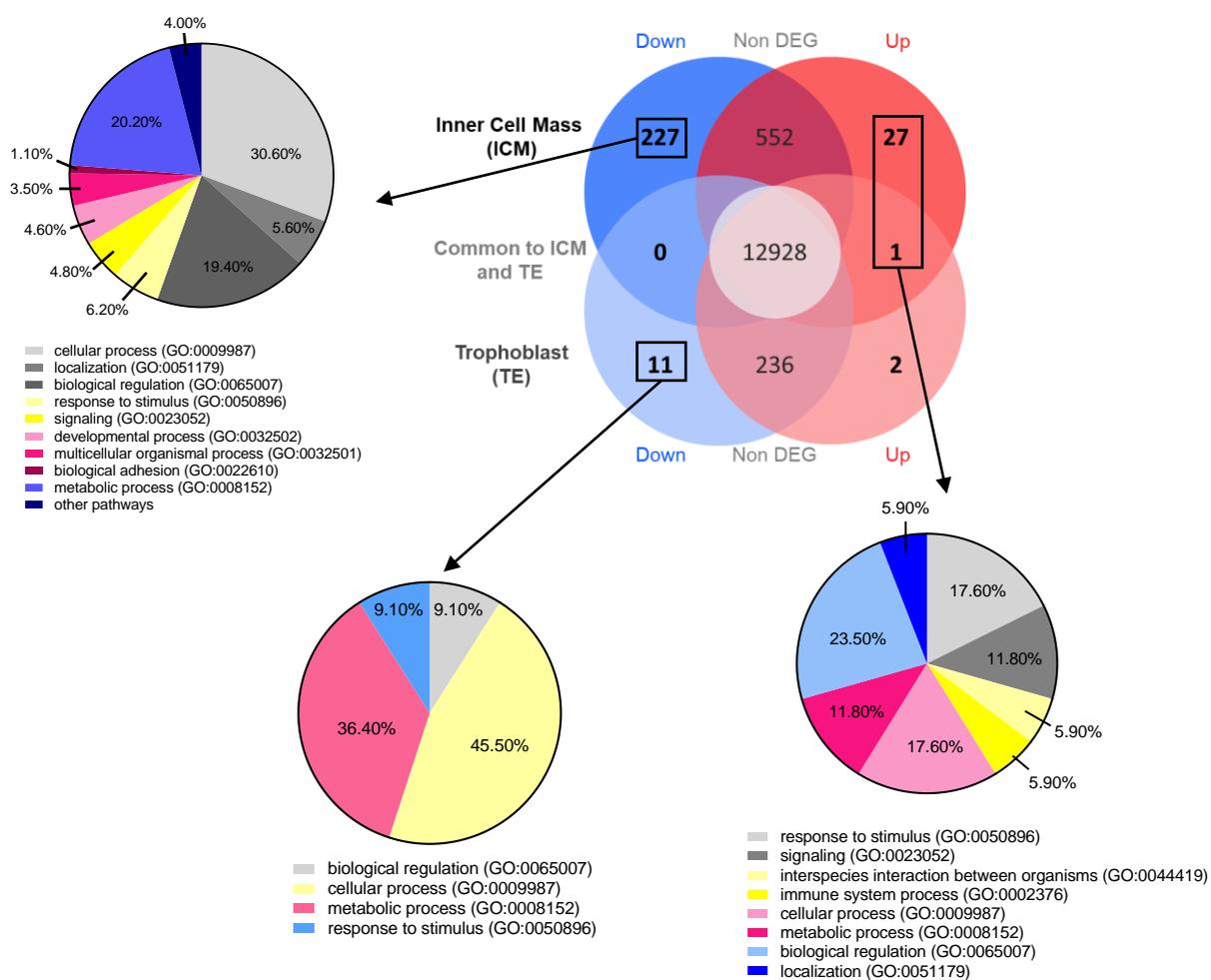
DeMixT\_ICM\_cells  
vs  
TE\_part

**B**

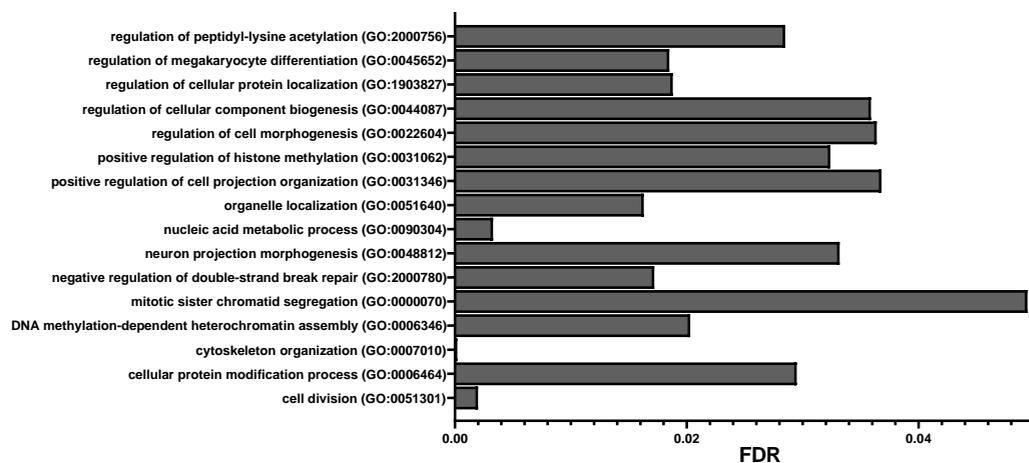
Individuals - PCA



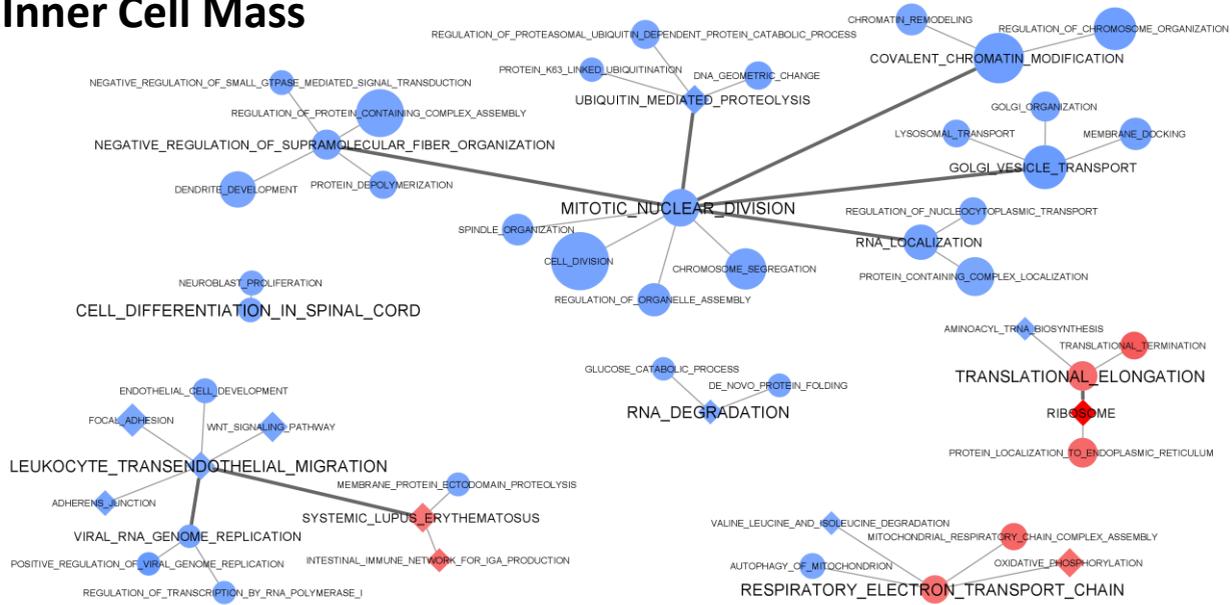
A



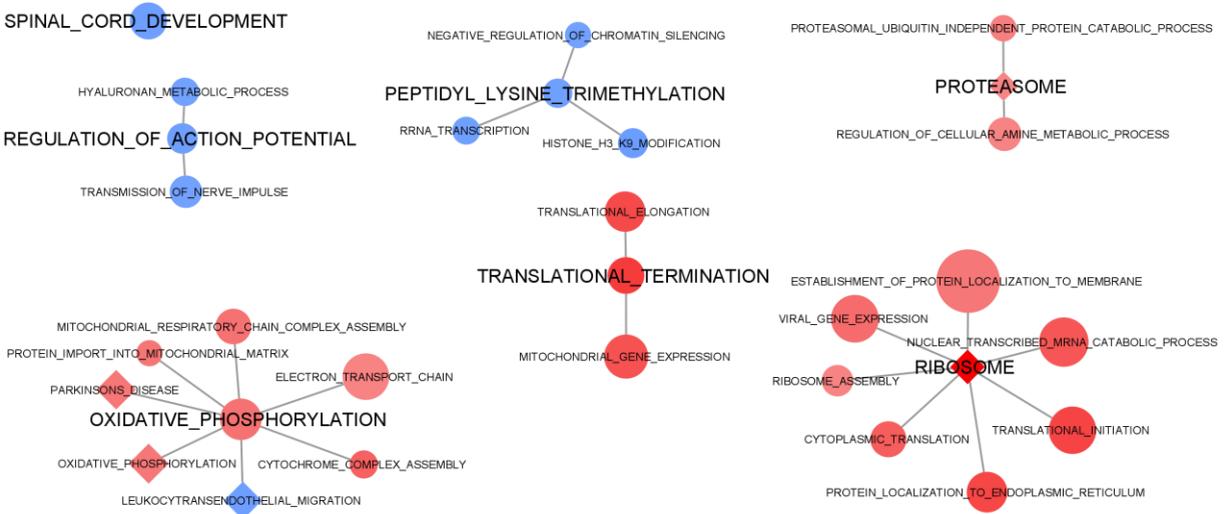
B



# Inner Cell Mass



# Trophoblast



# MATERNAL AGING

## Cell cycle / Mitotic division

- Assembly of spindle
- Chromosome segregation
- Cytokinesis/vesicle trafficking
- Catabolism of protein and mRNA

## Transcription

- Chromatin modification

## Cell signaling & adhesion

- Wnt signaling pathway
- Focal adhesion
- Adherens junction

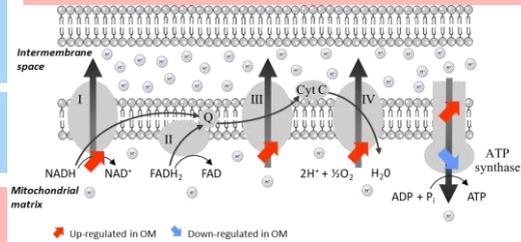
## Embryo development

- Cell fate commitment

## Translation

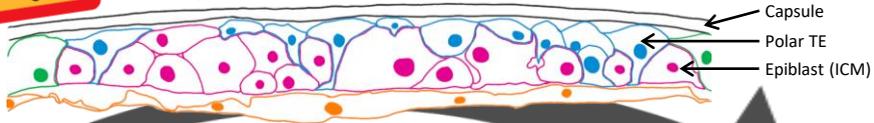
- Ribosome biogenesis

## Mitochondrial function



## ALTERED ICM DEVELOPMENT

Normal ICM development



The polar TE becomes discontinuous → isolated cells are still healthy and dividing and adhere to the epiblast  
The epiblast grows → cells are transcriptionally active and ingest polar TE cells by phagocytosis

Day 7

Day 8

Day 9

The mural TE divides following blastocyst growth  
The endoderm develops to be continuous and fully surround the blastocoel, with some areas too thin to be seen

Normal TE development



## ALTERED TE DEVELOPMENT

## Ion movement

- Transmission of action potential

## Transcription

- Chromatin modification

## Embryo development

## Protein catabolism

- Proteasome

## Mitochondrial function

## Translation

- Ribosome biogenesis

# MATERNAL AGING