Nulliparity affects the expression of a limited number of genes and pathways in Day 8 equine embryos

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Abstract

Nulliparous mares produce lighter and smaller foals compared to mares having previously foaled, with effects observed at least until 4 months of age. The need for a first gestation priming for the uterus to reach its full capacity has been proposed to explain this observation. Embryo developmental defects could be hypothesized but effects of maternal parity on the embryo have only been described once, in old mares, thus combining effects of parity and old age. The aim of this study was to determine effects of mare parity on embryo gene expression. Day-8 post ovulation blastocysts were collected from young (5/6 years old) nulliparous (YN, N=6) or multiparous (YM, N=4) non-nursing Saddlebred mares, inseminated with the semen of one stallion. Pure (TE_part) or inner-cell-mass-enriched (ICMandTE) trophoblast were obtained by embryo bisection for RNA sequencing (paired end, non-oriented, Illumina, NextSeq500). Deconvolution was performed on the ICMandTE dataset. Differential expression, with embryo sex and diameter as cofactors and gene set enrichment analysis (GO BP, KEGG, REACTOME databases) were performed using a false discovery rate <0.05 cutoff. Only a few genes were altered (ICM: n=18; TE: n=6) but several gene sets were perturbed (ICM: n=62; TE: n=50) by maternal parity. In YM, only pathways related to transcription, RNA processing and vesicle transport functions were enriched in the ICM whereas only pathways related to RNA localization were enriched in TE. In YN, while only gene sets related to ribosomes and extracellular matrix were enriched in the ICM, functions related to energy and lipid metabolism, lipid transport and interleukin-1 signaling were enriched in the TE. In conclusion, several genes and pathways are affected in embryos collected from nulliparous mares, with different effects on TE and ICM. Embryo development is altered in nulliparous mares, which could partially explain the term phenotype. Whether differences in gene expression result/induce poor embryo-maternal communication remains to be determined.

54 **Keywords:**

55 Blastocyst; RNA sequencing; horse; mare; periconception; equine

56

1. Introduction

In mammalian species, including the horse, it is now well established that the periconceptional and gestational maternal environment affect intra and extra-uterine growth and offspring long-term health [1,2]. These observations fall within the context of the Developmental Origins of Health and Diseases (DOHaD).

In horses, maternal parity defined as the number of gestations that produced a viable fetus (live or stillborn foal), is one of the main factors affecting the foal intra-uterine development. Indeed, foals born to primiparous mares (mares that have not foaled before) are lighter and smaller at birth and remain smaller until 18 months and lighter until 4 month of age compared to controls born to multiparous dams [3–13]. Their insulin sensitivity is higher than that of foals born to multiparous mares, and these data suggest that the normal decrease in insulin sensitivity observed in relation with foal age is delayed [13]. Similarly, testicular maturation is also delayed in foals born to primiparous mares [13]. These alterations in morphology and physiology of foals born to primiparous dams seem to be related to poorer performances in show jumping or on the racecourse than those of subsequent foals born to the same mare [14,15].

For a long time, these differences in mares' first born foals have been attributed to the need for a first gestation priming for the uterus to be able to reach its optimal size and vascularisation and fully support feto-placental developmental needs [16]. Indeed, primiparous mares produced lighter and less voluminous placentas than multiparous ones [8,12,13,16]. In horses, placentation is diffuse and the epitheliochorial placenta is in contact with the entire surface of the uterus [17,18]. Most feto-maternal exchanges occur through branched vascular structures that form interdigitations with the mare endometrium, called microcotyledons, that maximize nutrient exchanges by increasing feto-maternal contact surface [17–19]. Reduced placental volume and weight are associated with reduced foal development in first born foals and suggest that primiparity could be a form of intra-uterine growth restriction in horses.

The placenta derives from the equine embryo trophoblast. Its later efficacy is conditioned by proper implantation and development. Implantation takes place around 35-38 days post ovulation [20]. Prior to that, the equine embryo develops free in the uterus and depends on direct support of uterine secretions for its development. Impaired pre-implantation development in nulliparous mares could play a role in the reduced size of both term placenta and newborn foal. The few existing studies that consider maternal parity on fertility effects are controversial. While some found that parity did not affect fertility [21–27], others reported that mares that have never foaled have reduced embryo and fetal mortality compared to mares that previously foaled [27–33]. Confounding effects of maternal parity and age is probably the source of those discrepancies. Indeed, in a recent epidemiological study considering the effect of parity only in mares older than 10 years, there is a cumulative negative effect of nulliparity and aging on the rates of pregnancy at 14 days post-ovulation (ED and PCP, personal communication). Maternal age have been shown to affect oocyte and embryo developmental capacities (for review [34]) as well as gene expression in Day 8 embryos [35]. At the opposite, only one study considered the effect of maternal parity on preimplantation embryo and showed alterations of the expression of genes related to embryo development and exchanges with the environment were observed [36]. This study, however, only considered mares older than 10 years, in which uterine degenerative changes had probably occur. As maternal age affects embryo gene expression, it is important to consider maternal parity in young mares. At this time, there is no study considering the effect of parity on gene expression of embryos in young mares.

The aim of this study was to determine the effect of maternal nulliparity in young mares on embryo gene expression at the blastocyst stage. Young (5-6 years old) nulliparous and multiparous mares were inseminated with semen of the same stallion. Day-8 blastocysts were collected, measured and bisected to separate the pure trophoblast (TE_part) from the inner cell mass enriched hemi-embryo (ICMandTE). Gene expression was analyzed by RNA-seq in each compartment.

2. Materials and methods

2.1. Ethics

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

2.2. Embryo collection

Twenty-one non-nursing mares (mostly French Anglo-Arabian with some Selle Français) aged from 5 to 6 years old were included in this study. Mares were allocated to one of 2 groups according to their parity: nulliparous (YN, n = 10) and multiparous mares (YM, n = 11). Multiparous mares were defined as dams that had already foaled at least once while nulliparous mares were defined as mares that had never foaled before the experiment. During the experimental protocol, mares were managed in one herd in natural pastures 24h/day with free access to water with no nutritional supplementation but for salt blocks. The experiments took place from April 1st to May 3rd, 2019. All mares remained healthy during this period. During the experimentation, mare's withers' height and weight were measured. Characteristics of all mares and mares that produced an embryo are detailed in Table 1.

Mares were monitored as previously described [35]. Briefly, the mares' estrous period was monitored routinely by ultrasound with a 5MHz trans-rectal transducer. During estrus, ovulation was

induced with a single injection of human chorionic gonadotropin (i.v.; 750 - 1500IU; Chorulon® 5000; MSD Santé animale, France) as soon as one ovarian follicle >35mm in diameter was observed, together with marked uterine edema. Ovulation usually takes place within 48h, with > 80% occurring 25 to 48h after injection [37,38]. At the same time, mares were inseminated once with fresh or fresh overnight cooled semen containing at least 1 billion motile spermatozoa from a single fertile stallion. Ovulation was confirmed within the next 48 hours by ultrasonography.

Embryos were collected by non-surgical uterine lavage using prewarmed (37°C) lactated Ringer's solution (B.Braun, France) and EZ-Way Filter (IMV Technologies, France) 10 days after insemination, i.e., approximately 8 days post ovulation. Just after embryo collection, mares were treated with luprotiol an analogue of prostaglandin F2α (i.m; 7.5 mg; Prosolvin, Virbac, France).

The aim of the embryo collection was to obtain 5 embryos/group with each embryo coming from a different mare. Therefore, some mares that failed to produce an embryo at their first attempt were bred again for a second attempt.

2.3. Embryo bisection and RNA extraction

Using a binocular magnifying glass, collected embryos were immediately photographed with a size standard to subsequently determine embryo diameter using ImageJ® software (version 1.52a; National Institutes of Health, Bethesda, MD, USA). Embryos were then washed 4 times in commercially available Embryo holding medium (IMV Technologies, France) at 34°C and bisected with a microscalpel under binocular magnifying glass to obtain a trophoblast (TE_part) and an inner cell mass enriched (ICMandTE) hemi-embryo. At this stage, the TE_part is composed of trophectoderm and endoderm whereas the ICM is composed of epiblast layered on the internal side by endoderm cells [39,40]. Immediately after bisection, RNA extraction of each hemi-embryo was started in extraction buffer (PicoPure RNA isolation kit, Applied Biosystems, France) for 30 min at

42°C prior to storage at -80°C. RNA was extracted later from each hemi-embryo using PicoPure RNA isolation kit (PicoPure RNA isolation kit, Applied Biosystems, France), which included a DNAse treatment, following the manufacturer's instructions. RNA quality and quantity were assessed with the 2100 Bioanalyzer system using RNA 6000 Pico kit (Agilent Technologies, France) according to the manufacturer's instructions.

2.4. RNA sequencing

Five nanograms of total RNA were mixed with ERCC spike-in mix (Thermofisher Scientific, France) according to manufacturer's recommendations. Messenger RNAs were reverse transcribed and amplified using the SMART-Seq V4 ultra low input RNA kit (Clontech, France) according to the manufacturer recommendations. Nine PCR cycles were performed for each hemi-embryo. cDNA quality was assessed on an Agilent Bioanalyzer 2100, using an Agilent High Sensitivity DNA Kit (Agilent Technologies, France). Libraries were prepared from 0.15 ng cDNA using the Nextera XT Illumina library preparation kit (Illumina, France). They were pooled in equimolar proportions and sequenced (Paired end 50-34 pb) on NextSeq500 instrument, using a NextSeq 500 High Output 75 cycles kit (Illumina, France). Demultiplexing was performed with bcl2fastq2 version 2.2.18.12 (Illumina, France) and adapters were trimmed with Cutadapt version 1.15 [41]. Only reads longer than 10pb were kept.

2.5. RNA mapping and counting

As previously described [35], alignment was performed using STAR version 2.6 [42] on previously modified Ensembl 99 EquCab3.0 assembly and annotation. Genes were then counted with FeatureCounts [43] from Subreads package version 1.6.1.

179 ### *2.6. Data analysis*

180 All statistical analyses were performed by comparing YN to YM (YM set as reference group) using R
181 version 4.0.2 [44] on Rstudio software version 1.3.1056 [45].

182 Embryo were sexed using *X Inactive Specific Transcript* (*XIST*) expression as previously described [35].
183 Six embryos were determined as female (2 in the YN group and 4 in the YM group) while 5 were
184 considered as male (4 in the YN group, and 1 in the YM group).

185

186 #### ***2.6.1. Embryo recovery and fertility rate, embryo diameter and total RNA content***
187 ***analysis***

188 Embryo recovery rates (ERR) per mare and per ovulation were calculated as the number of attempts
189 with at least one embryo collected/total number of attempts. Both were analyzed using the Exact
190 Fisher test to determine if maternal parity influenced embryo recovery.

191 For total RNA content analyses, as embryos were bisected without strict equality for each hemi-
192 embryo, a separate analysis of ICMandTE and TE_part RNA quantities would not have been
193 meaningful. Thus, ICMandTE and TE_part RNA quantities were summed up. RNA quantity and
194 embryo diameter were analyzed using a linear model of nlme package version 3.1-148 [46] including
195 maternal parity and embryo sex, followed by 1000 permutations using PermTest function from
196 pgirmess package version 1.6.9 [47]. Variables were kept in the subsequent models when statistically
197 significant differences were observed. Differences were considered as significant for $p < 0.05$.

198

199 #### ***2.6.2. Deconvolution of gene expression in ICMandTE using DeMixT***

200 The deconvolution method has already been described in equine embryos [35]. Briefly, this method
201 enables the estimation of the relative gene expression of TE and ICM cell types within the hemi-

202 embryo ICMandTE which is composed of both trophoblast and inner cell mass in unknown relative
203 proportions. After filtering all genes with 3 non-null count values in at least one group (YN or YM) per
204 hemi-embryo (ICMandTE or TE_part), removing genes with a null variance in TE_part and adding the
205 value "1" to all count values in ICMandTE and TE_part datasets, deconvolution was performed using
206 the DeMixT R package version 1.4.0 [48,49]. Output datasets were DeMixT_ICM_cells and
207 DeMixT_TE_cells, corresponding to the deconvoluted gene expression in ICM cells and TE cells of
208 ICMandTE, respectively.

209 At the end of deconvolution, a quality check was automatically performed by the DeMixT R package
210 with the TE_part used as reference for DeMixT_TE_cells. Genes were automatically filtered out if the
211 difference between average deconvoluted expression of reference cells in mixed samples and
212 average expression of reference cells > 4.

213 Outputs of DeMixT_ICM_cells *vs* DeMixT_TE_cells, DeMixT_ICM_cells *vs* TE_part and ICMandTE *vs*
214 TE_part were compared with Deseq2 version 1.28.1 [50] to confirm that the deconvolution was
215 effective at separating gene expression. To check if deconvolution was efficient, as previously
216 described [35], the expression of several genes proper to ICM and TE cells in equine embryos
217 identified using literature search [51] was compared before and after deconvolution. Results of these
218 analyses were represented through manually drawn Venn diagrams as well as principal component
219 analysis graphics of individuals, using ggplot2 version 3.3.3 [52] and factoextra version 1.0.7 [53].

220

221 ***2.6.3. Maternal parity comparison for gene expression***

222 All genes with an average expression <10 counts in both YN and YM per hemi-embryo (ICM or TE)
223 were filtered out on the DeMixT_ICM_cells and TE_part datasets. Differential analyses were
224 performed with Deseq2 version 1.28.1 [50] with the YM group as reference, without independent
225 filtering. Genes were considered differentially expressed (DEG) for FDR <0.05 after Benjamini-
226 Hochberg correction (also known as false discovery rate, FDR). As ovulation was checked only every

48h and because embryos growth is exponential in the uterus, embryo diameter was considered as a cofactor in the model as well as embryo sex.

Equine Ensembl IDs were converted into Human Ensembl IDs and Entrez Gene names using gorth function in gprofiler2 package version 0.1.9 [54]. Genes without Entrez Gene names using gprofiler2 were manually converted when Entrez Gene names were available, using Ensembl web search function [55]. GO molecular function and GO Biological process annotations of genes were obtained from Uniprot website.

2.6.4. Gene set enrichment analyses (GSEA)

After log transformation using RLOG function of DESeq2 version 1.28.1, gene set enrichment analyses (GSEA) were performed on expressed genes using GSEA software version 4.0.3 (Broad Institute, Inc., Massachusetts Institute of Technology, and Regents of the University of California) [56,57] to identify biological gene sets disturbed by maternal parity. Molecular Signatures Databases [58] version 7.1 (C2: KEGG: Kyoto Encyclopedia of Genes and Genomes; REACTOME, C5: BP: GO biological process) were used to identify most perturbed pathways. Pathways were considered significantly enriched for FDR< 0.05. When the normalized enrichment score (NES) was positive, the gene set was enriched in the YN group while when NES was negative, the gene set was enriched in the YM group.

If applicable, as previously described in equine embryos [35], enriched terms from GO BP, KEGG and REACTOME databases were represented using SUMER analysis from SUMER R package version 1.1.5 and using FDR q-values [59]. Results were represented with graphs modified using Cytoscape version 3.8.2 [60]. In these graphs, gene sets are represented by nodes and the gene set size is represented by the size of the node. Node shape represents the gene set database (GO BP, KEGG or REACTOME). Blue nodes represent gene sets enriched in YN (NES > 0) while green nodes represent gene sets enriched in YM (NES < 0). Edge width represents the level of connection between representative

gene sets (thinner edges represent the first clustering while thicker edges represent the second clustering of the affinity propagation algorithm).

As SUMER is not able to consider only genes that participate to enrichment in GSEA, pathways with genes in common are grouped together, although genes in common are not the ones that participate to the enrichment. To better understand groups, therefore, authors, first recovered genes that were enriched in each pathway of a common group of gene sets according to SUMER analysis. Then, they only considered genes in common between pathways in one group to better qualify the function that was altered by maternal parity.

3. Results

3.1. Embryo recovery rates, diameter, total RNA content and quality and progesterone concentrations

Altogether, 25 embryo collections were performed (13 in YN and 12 in YM, 4 mares being flushed twice) and 12 embryos were obtained (7 from 6 YN mares and 5 from 5 YM mares). One young nulliparous mare produced twin embryos.

Embryo recovery rate per mare was 46% and 42% in YN and YM, respectively and did not differ between groups ($p = 1$).

Altogether, 1 and 2 double ovulations were observed, respectively, in YN and YM. The embryo recovery rate per ovulation at the time of embryo collection was not different according to group (50% in YN and 36% in YM, $p = 0.70$).

All embryos were expanded blastocysts grade I or II according to the embryo classification of McKinnon and Squires [61]. For the twin collection, embryos diameters were 580µm and 591µm. As only one embryo per mare was required, the 580µm diameter was randomly chosen for further analysis. Altogether, only 6 YN and all 5 YM embryos collected were RNA sequenced. Embryo diameter ranged from 457µm to 2643µm, with no effect of group on embryo diameter ($p = 0.18$). In

276 embryos selected for RNA sequencing, there was no effect of embryo sex on its size (p = 0.63). RNA
277 yield per embryo ranged from 12.0 ng to 2915.5 ng and was not related to parity (p = 0.07) nor
278 embryo sex (p = 0.77).
279 The median RNA Integrity Number (RIN) was 9.6 (8.9 - 10 range). Between 39.7 and 69.5 million
280 reads per sample were obtained after trimming. On average, 70.94% of the reads were mapped on
281 the modified EquCab 3.0 using STAR and 66.45% were assigned to genes by featureCounts.
282
283 *3.2. Deconvolution of gene expression to discriminate ICM and TE gene expression in*
284 *ICMandTE hemi-embryos*
285 After selecting genes with more than 3 non null count values in at least one group (YN or YM) per
286 hemi-embryo (ICMandTE or TE_part), 16,901 genes were conserved for deconvolution. In addition,
287 67 genes were removed because their variance was null in the TE_part. For these genes, the mean
288 count in ICMandTE samples was above 110 counts. The deconvolution quality of all gene was
289 sufficient. Therefore, at the end of the deconvolution algorithm, 16,834 genes were available for
290 differential analysis.
291 Before deconvolution, 681 genes were differentially expressed (FDR < 0.05) between the ICMandTE
292 and the TE_part (Fig. 1a). After deconvolution, the comparison between DeMixT_ICM_cells and
293 DeMixT_TE_cells yielded 6,171 differentially expressed genes while the comparison
294 DeMixT_ICM_cells *vs* TE_part yielded 5,262 differentially expressed genes, with 4713 genes in
295 common with the previous comparison (70%). Moreover, 677 of the initially 681 differentially
296 expressed genes before deconvolution were also identified as differentially expressed in both post-
297 deconvolution analyses. Only in the comparison DeMixT_ICM_cells *vs* TE_part, 3 among the 4
298 remaining genes were identified. On the PCA graph of individuals, ICMandTE and TE_part were partly
299 overlapping (Fig 1b). DeMixT_TE_cells and TE_part superposed well, suggesting that datasets before
300 and after deconvolution have a similar global gene expression; whereas the DeMixT_ICM_cells group
301 is clearly separated from others on Axis 1 (22.3% of variance), indicating that the deconvolution

effectively enabled the separation of gene expression of the two cell types in the mixed part (ICMandTE).

On the 12 genes previously identified by Iqbal et al. as more expressed in the ICM [51], one had to be removed before deconvolution because its variance in the TE was zero (*ENSECAG00000010653*, annotated as SRY-Box Transcription Factor 2, *SOX2*). On the 11 remaining genes, 4 were also more expressed in the ICMandTE *vs* TE_part comparison (Table 2). After deconvolution (comparison DeMixT_ICM_cells *vs* TE_part), 10 out of 11 of these genes were effectively more expressed in the ICM. Iqbal et al. identified 7 genes that were more expressed in the TE. One of those genes was differentially expressed in the comparison ICMandTE *vs* TE_part, *i.e.*, before deconvolution. After deconvolution, the expression of 3 of the 7 reported genes, different from the only gene identified before deconvolution, were increased in the TE_part compared to the DeMixT_ICM_cells.

All of these results validate the deconvolution procedure and justify the use of data from the DeMixT_ICM_cells file. In the following results, the TE_part was used as representative of TE and DeMixT_ICM_cells was used as representative of gene expression in the ICM.

3.3. Sample selection

One embryo (YM) was larger than 2000 µm while all other embryos were smaller than 1400µm in diameter (Supplementary Figure 1). Embryo size has been shown to affect equine embryo gene expression [62]. Thus, the analysis was performed both with or without this large embryo to check if results were affected. All but one differential expressed genes identified with the largest embryo were also found differentially expressed without it (Supplementary Figure 2). Nevertheless, to limit size effect, the analyses described below are those without the largest embryo, where only 6 YN and 4 YM embryos were analyzed. The results of the differential analyses that were performed including the 2643µm large YM embryo are shown in Supplementary Tables 1 and 2.

3.4. Differential gene expression in deconvoluted ICM cells

After retaining only genes with an average expression ≥ 10 counts in at least one maternal parity group andhemi-embryo, 14,418 genes were considered as expressed in the YN or YM embryos ICM cells. Only 18 genes were differentially expressed (12 downregulated and 6 upregulated in YN) (Fig. 2 and Supplementary table 3). Respectively, 11 and 5 genes out of the down- and upregulated genes were associated to a protein known and described in human. These 16 genes an gene sets determined from Uniprot in wich they are susceptible to play a role are presented in Table 3.

3.5. Differential gene expression in the TE part

In the TE, 13,203 genes were considered as expressed in YN or YM. Only 6 were differentially expressed (Supplementary table 4) with half being down and up- regulated in YN (Fig. 2). Except one that was a long noncoding RNA, all other genes were associated to a known protein in human. These genes are presented in Table 4 with the pathways in which they are susceptible to play a role.

3.6. Gene set enrichment analysis in deconvoluted ICM cells

After Entrez Gene ID conversion, 12,892 genes were considered expressed in ICM cells. Fifty-eight GO Biological Process and 4 KEGG pathways were disturbed by maternal parity in ICM cells (Supplementary table 5). After SUMER analysis, 2 and 27 gene sets, respectively enriched in YN and YM, were represented (Fig. 3). They were clustered in 8 groups. The group enriched in YM and clustered under the term “DNA recombination” was composed of genes related to the maintenance of DNA integrity, chromosome segregation and recombination. Enriched in YM groups “NCRNA metabolic process” and ‘Peptidyl lysine trimethylation” contained both, genes related to methylation and transcription. The only gene set enriched in YN in the group “NCRNA metabolic process” was mainly enriched by genes encoding for a subunit of ribosomes that were common with other gene sets enriched in YM. Genes related to ribosomes were, however, not participating in gene set enrichment of other pathways in this cluster. “multi organism localization”, “RNA splicing” and “RNA localization” clusters were composed of genes involved in RNA maturation and transport. The last

group enriched in YM was clustered under the term "vesicle targeting" and was containing genes related to intracellular transport. In this group, the pathway "Golgi vesicle transport" included a DEG that was Vacuolar Protein Sorting-Associated Protein 52 Homolog (*VPS52*), up-regulated in the ICM of embryos from YM mares. The only one group enriched in YN was composed of one pathway named "ECM receptor interaction" in which genes related to extracellular matrix (ECM) were observed.

3.7. Gene set enrichment analysis in TE

After Entrez Gene ID conversion, 11,889 genes were considered expressed in TE from YN or YM embryos. Altogether, 50 gene sets from GO BP, KEGG and REACTOME were perturbed (23 GO BP, 7 KEGG and 20 REACTOME) by maternal parity in young mares (Supplementary table 6). After SUMER analysis, 36 gene sets were represented (Fig. 3) and were clustered in 8 groups. Among them, 7 were enriched in YN. The first group was composed of one gene set named "cardiac septum morphogenesis". Most genes that participated to the enrichment of this pathway were related to transcriptional factors. The second group was composed of 3 pathways and clustered under the term "negative regulation of secretion". Genes that participated most to the enrichment of these pathways were related to the innate immunity, more particularly to the production and transport of the interleukin 1 beta (IL1B). Altogether, 3 groups were related to the production of energy inside the cell. Indeed, the cluster under the term "Parkinsons disease" was actually composed of genes with an enriched expression that were related to oxidative phosphorylation. One group was "Hydrogen peroxide metabolic process" where genes that participated the most to the enrichment were involved in the degradation of hydrogen peroxide. The last group involved in energy production was clustered under the term "valine, leucine and isoleucine degradation". Genes that participated the most to the enrichment of these pathways were directly involved in the beta oxidation of fatty acid. The group under the term "positive regulation of lipid transport" was composed of genes related to the regulation of the transport of lipids and cholesterol. The last group enriched in YN was

clustered under the term "eukaryotic translation elongation" and was mostly enriched due to components of the ribosomes that mostly participate in these enrichments. Moreover, the REACTOME pathway "RRNA modification in the nucleus and cytosol" was clustered with this group because of genes that encoded for ribosome components. These genes were, however, not enriched in this particular pathway. Genes that participate to its enrichment, nevertheless, were related to ribosome biogenesis. The only one group enriched in YM was represented by the term "Regulation of glucokinase by glucokinase regulatory protein". These pathways were mostly enriched in YM because of genes that encode for nucleoporin subunits.

4. Discussion

Maternal parity in young mares slightly affected both ICM and TE gene expression without affecting embryo recovery rates nor growth. Although only a few genes were affected by maternal parity, up regulated genes in the ICM of embryos from young nulliparous were involved in lipid, amine and creatinine metabolism, positive regulation of transcription, growth factor signaling and morphogenesis while down regulated were related to extracellular matrix (ECM) disassembly, reactive oxygen species (ROS) metabolism, transcription regulation, endocytosis, protein transport, protein metabolism, MAP kinase signalization and cell differentiation. In the TE, only five known genes were observed differentially expressed. One of the 3 up regulated genes in the TE of embryos from YN was involved in the cell response to hypoxia while the 2 others encode for ion binding proteins. Down regulated genes in the TE of embryos from YN mares were related to prostaglandin metabolism and amino acid/creatinine exchange. Interestingly, while gene set enrichment analysis showed almost only pathway enrichment in YM in the ICM, in the TE, almost all pathways were enriched in YN embryos. In the ICM, after SUMER analysis, the pathways enriched in YM were related to DNA modification, RNA production and maturation and cell transport while gene sets related to the extracellular matrix function and ribosome were enriched in ICM of YN embryos. In the TE, gene

405 sets enriched in embryos from YN were related to immunity, growth factor signaling,
406 phosphorylation oxidative, metabolism of reactive oxygen species, beta oxidation and transport of
407 lipids and ribosome while gene sets enriched in embryos from YM were related to nucleoporins.
408
409 In the ICM of embryos from YM mares, enriched gene sets were mostly related to DNA conformation
410 and methylation changes as well as RNA formation, transport and maturation. These results suggest
411 that transcription and regulating pathways are less active in embryos from nulliparous mares. These
412 results are comforted by the fact that teneurin transmembrane protein 3 (*TENM3*) is downregulated
413 in embryos from nulliparous mares. This gene is part of the teneurin family, which encodes for
414 transmembrane proteins that are essential for embryo morphogenesis and nervous system
415 development. The knockdown of these genes in mice and drosophila leads to embryo lethality (for
416 review [63]). Altogether, these results suggested that ICM growth and development would be poorer
417 in embryo from nulliparous compared to multiparous mares but, here, no difference in embryo size
418 had been observed. One hypothesis could be because only ICM seemed affected or because, embryo
419 size at a same age is highly variable as shown in several studies [64–68], although ovulation check
420 was performed twice daily [69]. This huge variation could hide size differences in studies.
421 In the TE, the gene named "family with sequence similarity 162 member A" (*FAM162A*), also known
422 as E2-Induced Gene 5 Protein (*E2IG5*) or growth and transformation-dependent protein (*HGTD-P*)
423 was up-regulated in embryos from nulliparous mares. This gene is one of the hypoxia inducible
424 factors (HIF)-activated downstream gene and is normally responsible of the activation of
425 mitochondrial proapoptotic cascades when overexpressed [70]. As energy production processes
426 (protein and lipid oxidation, oxidative phosphorylation and related regulatory pathways) were
427 enriched in embryos from nulliparous mares, it seemed unlikely that *FAM162A* up-regulation in
428 embryos from nulliparous mares was a response to hypoxic environment. Nevertheless, it could be
429 hypothesized that the uterine environment of embryos may vary according to mares' parity, partly
430 due to reduced uterine blood perfusion in nulliparous mares. To the authors' knowledge, there is no

study on the effect of nulliparity on uterine vascularization in young mares. It has nonetheless been shown that *FAM162A* expression is increased in intestinal and uterine cervical cancer [71,72] its overexpression enhanced cell proliferation processes, suggesting a non-elucidated positive role in tumor development [72]. As in tumor, here, *FAM162A* could play a role in cell proliferation of equine embryos but the process remains to be elucidated. This could also indicate that proliferation in equine embryos differ according to mares' parity, maybe as a response to their environment.

In the TE of embryos from nulliparous mares, pathways related to oxidative phosphorylation were enriched. The enrichment of the expression of genes involved in these pathways could indicate that the production of ATP from oxidative phosphorylation is up regulated in TE of embryos from nulliparous mares in comparison to the ones from multiparous mares. This up-regulation of oxygen oxidation in mitochondria could be harmful for TE cells as oxidative phosphorylation is accompanied by the production of reactive oxygen species (ROS) and particularly of hydrogen peroxide (for review [75]). Pathways related to hydrogen peroxide metabolic processes, however, were also enriched in the TE of embryos from nulliparous mares, showing that there is an up-regulation of the control of ROS such as hydrogen peroxide. The up regulation of both oxidative phosphorylation and regulation of ROS pathways suggests that there is an increased production of energy that is not harmful because well controlled in the TE of embryos from nulliparous mares.

At this developmental stage in equine embryos, 40 to 50% of glucose uptake is oxidized in the mitochondria, probably to meet the high energy demand of ionic transport associated with the important growth of both blastocoelic cavity and trophoblast [76]. Here, however, the enrichment in oxidative phosphorylation was not accompanied by an enrichment in glucose metabolism nor transport pathways but pathways linked to beta-oxidation of lipids and degradation of amino acids were enriched. This suggests that glycolysis is not affected by maternal parity but, to meet energy requirements, embryos from nulliparous mares use fatty acids and/or amino acids whilst embryos from multiparous mares do not need more energy than already provided and therefore, do not require the degradation of these substrates. The increased catabolism of amino acids and lipids could

457 be detrimental for embryo development as the first are required for protein synthesis and the latter
458 are mandatory for hormone production (for review [77,78]). Pathways related to amino acid
459 degradation, however, were mostly enriched in genes involved in beta-oxidation of fatty acids.
460 Moreover, pathways related to translation and protein maturation but not pathways related to
461 amino acids transport were enriched in embryos from nulliparous mares. Altogether, these results
462 suggest that only lipid catabolism is enriched in the TE of nulliparous mares' embryos.
463 In addition, pathways related to the transport of lipids and cholesterol were enriched in the TE of
464 nulliparous mares' embryos compared to those of multiparous mares. One hypothesis to explain
465 these results could be that there is a higher energy demand in embryos from nulliparous mares and
466 that they would compensate by degrading more lipids for oxidative phosphorylation, which requires
467 more lipids to be obtained from the external environment. Another possibility is that the lipid
468 composition of the uterine environment is altered in nulliparous mares, possibly due to immature
469 uterine glands, leading to increased absorption by the embryo, that would stimulate beta-oxidation
470 and thus oxidative phosphorylation. Indeed, the metabolism of lipids was also shown to be perturbed
471 in blastocysts at the same developmental stage according to maternal parity in old mares [36]. To the
472 authors' knowledge, there is no study on the effects of maternal parity in any species on uterine fluid
473 composition and how it could interfere with embryo gene expression. Although it is more likely that
474 there are modifications in the uterine environment according to mare's parity, the present results
475 cannot conclude about the origin of the altered embryo metabolism.
476 As a confirmation of increased lipid transport, retinol binding protein 1 (*RBP1*) was up regulated in
477 the ICM of embryos from nulliparous mares. Retinol is well known to be an important regulator of
478 vertebrate development (for review [79]). In bovine, the addition of retinol to the maturation and
479 culture medium of oocytes and embryos increased the blastocyst rate [80]. RBP transports the
480 hydrophobic retinol in physiological fluids such as plasma [81] or uterine fluids. Pig conceptuses at
481 the time of elongation produce RBP in large amounts, suggesting that retinol is important for embryo
482 development [82]. In horses, the expression of *RBP* increased in the endometrium during diestrus

483 under steroid regulation but did not vary according to the presence of an embryo or not [83].
484 Although underlying mechanisms are missing, *RBP1* could, however, play an important role in equine
485 early embryo development by transporting retinol to the embryo. The increased expression of RBP in
486 the ICM of embryos from nulliparous mares could be a response to a reduced availability of retinol in
487 the close environment of the embryo or an increased requirements of retinol from embryos of
488 nulliparous mares.
489 Nutrient and ion exchanges were also modified by maternal parity in the TE. Indeed, solute carrier
490 family 47 member 1 (*SLC47A1* also known as *MATE1*), the solute transporter for molecules such as
491 creatinine or guanidine, was down regulated in the TE of embryos from nulliparous mares. In
492 addition, the expression of EF-hand calcium binding domain 11 (*EFCAB11*) and GTP binding protein 8
493 (*GTPBP8*) was increased in the TE of embryos from nulliparous mares compared to that of
494 multiparous mares. These results could indicate a perturbed transport of different molecules in the
495 TE of embryos from nulliparous mares.
496 These modifications of cell metabolism in the TE were associated with an alteration of pathways
497 related to immunity, especially those linked to interleukin 1 beta (IL1B), being enriched in embryos
498 from nulliparous mares. In cattle, it has been suggested that the early bovine embryo interacts with
499 the dam's immune system through processes involving IL1 [84]. In horses, maternal recognition of
500 pregnancy (MRP) is thought to take place between 10-13 days post ovulation (for review [85]). At 19-
501 and 25-days, but not at 13 days post ovulation, expression of the *IL1 receptor antagonist* has been
502 shown to be markedly increased in the endometrium of pregnant compared to cyclic mares,
503 suggesting that the endometrium regulates the IL1 signal and that IL1 plays a role in MRP in equine
504 [86]. The expression of *IL1B* is increased in the luminal epithelium of pregnant *vs* cyclic mares at 10-
505 13 days post ovulation, confirming the involvement of *IL1B* signaling process in MRP [87]. Here,
506 embryos were collected earlier from the assumed MRP period but the observed differences in the
507 IL1B signaling pathway could indicate that embryo-maternal communication and possibly MRP are
508 affected by maternal parity.

509 Furthermore, related to lipid metabolism and IL1B signaling, peroxiredoxin like 2B (*PRXL2B*), also
510 known as Prostamide/Prostaglandin F Synthase, was downregulated in the TE of nulliparous mares'
511 embryos. This gene encodes for an enzyme that has been shown to catalyze the reduction of
512 prostamide H_2 to prostamide $F_{2\alpha}$ as well as the reduction of prostaglandin H_2 (PGH_2) to prostaglandin
513 $F_{2\ \alpha}$ ($PGF_{2\alpha}$) [88]. In bovine, IL1B upregulates $PGF_{2\alpha}$ and prostamide secretion by in vitro cultured
514 endometrial cells [89]. In horses, $PGF_{2\alpha}$ is secreted by the uterus to provoke the corpus luteum
515 luteolysis (for review [90]). It has been shown that the suppression of the pulsatile secretion of $PGF_{2\alpha}$
516 from the endometrium is responsible for the maintenance of pregnancy [91] and that *in vitro*, $PGF_{2\alpha}$
517 production is significantly reduced when endometrial explants are co-cultured with embryonic
518 tissues [92]. From the oviduct stages, equine embryos are able to produce prostaglandins [93–95].
519 Prostaglandins produced by the embryo, however, do not reach the blood circulation in sufficient
520 amount to induce luteolysis [91]. It has been shown that these prostaglandins are required for
521 myometrial contractions that participate in the migration of the equine embryo at the time of MRP
522 [96]. By impeding the movement of the embryo, one study observed that equine embryo migration
523 through at least 2/3 of the uterus is required to prevent luteolysis [97]. Moreover, the use of an
524 intra-uterine device to imitate the physical presence of an embryo, allowed to prevent the luteolysis
525 [98]. A recent study, nevertheless, observed that the contact of a substance/object is not sufficient to
526 reduce PGF secretion from the endometrium, suggesting that embryo secretions are required for
527 luteolysis [99]. Therefore, although MRP is thought to begin 2 days later, the present study shows
528 that MRP might be delayed or disturbed in nulliparous mares.
529

530 ## 5. Conclusion

531 So far, the effect of mare's parity on embryo gene expression had never been considered. The
532 present study shows that mare's parity affects the expression of genes in both ICM and TE of
533 blastocysts. Only the expression of few genes is altered but several important functions for embryo

development are affected by mare's parity. Indeed, nulliparity in young mares particularly alters the expression of genes related to transcription and RNA processing in the ICM and embryo-maternal communication in the TE, suggesting embryo adaptation to an environment that is different in nulliparous *vs* multiparous mares. Individual chances of implantation for each embryo could not be predicted by the results of this study. Until today, the capacity of uterus to enlarge and support pregnancy was the only suggested explanation for the lighter and smaller foal and placenta at birth in nulliparous mares. The present results indicate differences in embryo-maternal communication long before implantation that could alter the embryo development as well as maternal recognition of pregnancy.

Data Availability Statement

The RNA sequençing data supporting the conclusions of this article are available in the GEO SuperSeries [accession: GSE193676; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE193676], containing repositories [accession: GSE162893; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE162893] and [accession: GSE193675; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE193675].

Conflict of interest

The authors declare no conflicts of interest.

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Author contributions

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

List of abbreviations

DEG: differential expressed genes

DeMixT_ICM_cells: deconvoluted gene expression in ICM cells

DeMixT_TE_cells: deconvoluted gene expression in TE cells

ECM: Extracellular matrix

ERR: embryo collection rate

FDR: false discovery rate

GO BP: Gene Ontology biological process

GO: Gene Ontology

GSEA: gene set enrichment analyses

ICM: inner cell mass

ICMandTE: inner cell mass enriched hemi-embryo

ICSI: intracytoplasmic sperm injection

IL1B: Interleukin 1 beta

KEGG: Kyoto Encyclopedia of Genes and Genomes

Log2FC: log2 fold change

NES: normalized enrichment score

OM: old multiparous mares

ON: old nulliparous mares

TE: trophoblast

593 TE_part: pure trophoblast hemi-embryo

594 XIST: X inactive Specific Transcript

595

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873

874 ## Figure legends

875 Fig. 1: Gene expression in ICM and TE before and after deconvolution using DeMixT

876 a) Venn diagram of genes differentially expressed in the different analyses: ICMandTE *vs* TE_part
877 (before deconvolution), DeMixT_ICM_cells *vs* DeMixT_TE_cells (after deconvolution) and
878 DeMixT_ICM_cells *vs* TE_part (gene expression of ICM after deconvolution *vs* gene expression in
879 TE_part without deconvolution); b) Principal Component Analysis of gene expression from
880 DeMixT_ICM_cells, DeMixT_TE_cells, ICMandTE and TE part datasets.

881 Deconvolution was used to isolate gene expression of ICM and TE cells in ICMandTE hemi-embryos.
882 ICMandTE: inner cell mass + trophoblast; TE_part: pure trophoblast. Here trophoblast represents
883 trophectoderm + endoderm.

884

885 Fig. 2: Analysis of differentially expressed genes (DEG) in embryos according to maternal parity

886 A) representation of down- (blue) and up- (red) regulated DEG in ICM (from DeMixT_ICM_cells data
887 obtained after deconvolution of ICMandTE using DeMixT R package [48,49]) and TE (from TE_part
888 dataset) of embryos from ON *vs* OM.

889 DEG: Differentially Expressed Genes (FDR < 0.05); TE: Trophoblast; ICM: Inner Cell Mass; ON: Old
890 nulliparous mares; OM: Old multiparous mares

891

892 Fig. 3: SUMER clustering of GSEA terms clustering of the most perturbed terms in the ICM and TE of
893 embryos according to mares' parity

894 Nodes represent altered gene sets in the ICM and TE (FDR <0.05). Node size represents the gene set
895 size. Node shape represents the gene set database: GO BP (circle) or KEGG (diamond) or REACTOME
896 (square). Gene sets are represented in blue if enriched (NES >0) in young nulliparous mares' embryos
897 and in green if enriched (NES <0) in young multiparous mares' embryos. The lighter the color, the
898 more the NES is close to 0. Edges represent the level of connection between representative gene
899 sets. This graph was performed using SUMER R package [59] and modified using cytoscape 3.8.2 [60]

900 GSEA: Gene set enrichment analysis; ICM: Inner cell mass; TE: trophoblast; FDR: False Discovery Rate;
901 GO BP: Gene Ontology Biological Process; Kyoto Encyclopedia of Genes and Genomes; NES:
902 Normalized Enrichment Score

Supplementary material

Supplementary Figure 1:

SupFig1_Embryosize.tif

Plot of equine individual embryo according to their size

Supplementary Figure 2:

SupFig2_comp_with_without.png

Venn diagrams of differential analyses on equine embryo gene expression according to maternal parity in the inner cell mass (ICM) and the trophoblast part (TE) with or without the largest embryo (2643µm in diameter)

Supplementary Table 1:

Sup1_ICM_Diff_avecYME5.csv

Differential gene analysis using DeSeq2 in DeMixT_ICM_cells of equine embryo at Day 8 post-ovulation according to mares' parity with the large embryo

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

ICM: Inner cell mass; YN: young nulliparous mares; YM: young multiparous mares

924 Supplementary Table 2:

925 Sup2_TE_Diff_avecYME5.csv

926 Differential gene analysis using DeSeq2 in TE_part of equine embryo at Day 8 post-ovulation
927 according to mares' parity with the large embryo

928 Equine ensemble ID, orthologue human Ensembl ID, Orthologue human Entrez Gene ID, gene
929 description, normalized counts for each embryo and parameters obtained after Deseq2 analysis
930 (log2FoldChange, pvalue and padj (after FDR correction)) of genes expressed in TE_part of YN and YM
931 embryos

932 TE: trophoblast; YN: young nulliparous mares; YM: young multiparous mares

933

934

935 Supplementary Table 3:

936

937 Sup3_ICM_Diff_sansYME5.csv

938 Differential gene analysis using DeSeq2 in DeMixT_ICM_cells of equine embryo at Day 8 post-
939 ovulation according to mares' parity without the largest embryo

940 Equine ensemble ID, orthologue human Ensembl ID, Orthologue human Entrez Gene ID, gene
941 description, normalized counts for each embryo and parameters obtained after Deseq2 analysis
942 (log2FoldChange, pvalue and padj (after FDR correction)) of genes expressed in ICM (after gene
943 expression deconvolution of ICMandTE using DeMixT) of YN and YM embryos

944 ICM: Inner cell mass; YN: young nulliparous mares; YM: young multiparous mares

945

946 Supplementary Table 4:

947 Sup4_TE_Diff_sansYME5.csv

948 Differential gene analysis using DeSeq2 in TE_part of equine embryo at Day 8 post-ovulation
949 according to mares' parity with the largest embryo

950 Equine ensemble ID, orthologue human Ensembl ID, Orthologue human Entrez Gene ID, gene
951 description, normalized counts for each embryo and parameters obtained after Deseq2 analysis
952 (log2FoldChange, pvalue and padj (after FDR correction)) of genes expressed in TE_part of YN and YM
953 embryos

954 TE: trophoblast; YN: young nulliparous mares; YM: young multiparous mares

955

956 Supplementary Table 5:

957 Sup5_ICM_GSEA_sansYME5.csv

958 Gene set enrichment analysis results on gene expression of DeMixT_ICM_cells of embryos from
959 young nulliparous and multiparous mares

960 Gene Set Enrichment Analysis results (database, pathway name, size, enrichment score without and
961 with normalization, p-value and FDR corrected q-value) for GO biological process, KEGG and
962 REACTOME databases on DeMixT_ICM_cells gene expression table. These results did not include
963 YM_E5, the embryo larger than 2,000µm. ICM: Inner cell mass

964

965 Supplementary Table 6:

966 Sup6_TE_GSEA_sansYME5.csv

967 Gene set enrichment analysis results on gene expression of TE_part of embryos from young
968 nulliparous and multiparous mares

969 Gene Set Enrichment Analysis results (database, pathway name, size, enrichment score without and
970 with normalization, p-value and FDR corrected q-value) for GO biological process, KEGG and
971 REACTOME databases on TE_part gene expression table. These results did not include YM_E5, the
972 embryo larger than 2,000µm.

973

Tables

Table 1: Mares' characteristics at embryo collection time.

	Nulliparous (YN)		Multiparous (YM)	
Characteristics	**Total (n = 10)**	**With embryo (n = 6)**	**Total (n = 11)**	**With embryo (n = 5)**
Breed	AA n = 7; SF n = 3	AA n = 5; SF n = 1	AA n = 9; SF n = 1; SB n=1	AA
Age *(in years)*	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
Parity *(number of foalings)*	0 ± 0.00	0 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Weight *(in kg)*	535.66 ± 30.72	544.55 ± 30.50	536.62 ± 44.12	524.02 ± 61.39
BCS *(scale 1-5)*	2.35 ± 0.32	2.21 ± 0.25	2.16 ± 0.28	2.3 ± 0.33
Withers' height *(in cm)*	159.70 ± 3.33	161.67 ± 2.50	157.95 ± 3.74	157.40 ± 5.41

AA: Anglo Arab or Anglo-Arabian type; SF: Selle Français section A or B; SB: Saddlebred. Age, parity, weight, and height are presented as mean ± SD

979 Table 2: Comparison of the expression of selected genes previously identified as specific to TE or ICM

980 in equine embryos [51], 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
ICM	**SOX2**	ENSECAG00000010653	-	-	-	-
	NANOG	ENSECAG00000012614	5.78	6.93E-58	7.09	9.24E-81
	SPP1	ENSECAG00000017191	4.52	2.21E-12	5.86	6.46E-21
	LIN28B	ENSECAG00000020994	3.21	6.84E-13	4.44	2.00E-26
	SMARCA2	ENSECAG00000024187	1.01	0.139	1.71	8.10E-05
	POU5F1 (OCT4)	ENSECAG00000008967	0.62	8.32E-04	1.25	1.22E-06
	ID2	ENSECAG00000008738	0.33	0.604	0.52	0.025
	DNMT3B	ENSECAG00000012102	0.49	0.056	1.04	5.66E-07
	DPPA4	ENSECAG00000013271	0.39	0.545	0.87	2.63E-04
	SALL4	ENSECAG00000018533	0.21	0.177	0.60	1.50E-16
	KLF4	ENSECAG00000010613	0.03	0.995	-0.39	0.526
	UTF1	ENSECAG00000039888	0.34	0.726	0.66	0.047
TE	**TFAP2A**	ENSECAG00000017468	-0.21	0.010	-0.07	0.42
	CDX2	ENSECAG00000027754	-0.21	0.290	-0.37	2.87E-04
	ELF3	ENSECAG00000014608	-0.12	0.864	-0.19	0.148
	GATA2	ENSECAG00000016768	-0.10	0.913	-0.05	0.75
	GATA3	ENSECAG00000024574	-0.18	0.28	-0.10	0.325
	TEAD4	ENSECAG00000011303	-0.19	0.206	-0.31	9.58E-06
	FREM2	ENSECAG00000020410	-0.06	0.970	0.13	0.474

981

982 Gene expressions were obtained from RNA of 11 equine embryos bissected in two hemi-embryos:

983 one part was composed only of trophoblast (TE), TE_part, while the other part was composed of TE

984 and inner cell mass (ICM), ICMandTE. As it is impossible to estimate the proportion of each cell in

985 ICMandTE, a deconvolution algorithm (package DeMixT) was used to estimate gene expression of

986 these different kind of cells. DeMixT_ICM_cells dataset corresponds to the deconvoluted gene

expression of ICM cells from ICMandTE. Log2 fold change (log2FC) and padj (adjusted p-value with Benjamini-Hochberg correction) were obtained with Deseq2 package. TE_part is the reference group in both analyses: when log2 fold changes (log2FC)>0, the gene is more expressed in the ICMandTE or DeMixT_ICM_cells, while when log2FC<0, the gene is more expressed in the TE_part.

Green is used to represent genes differentially expressed in the present study. Orange is used to represent genes that have been previously identified as predominant in the ICM [51] but that are identified here as predominant in the TE.

6 Table 3: Up- and down-regulated genes coding for a protein in the inner cell mass of equine embryos according to mare parity

Ensembl Name	Entrez Gene ID	Description	GO Molecular function	GO Biological Process	log2 Fold Change	padj
ENSECAG00000029895	*MAGEB16*	MAGE family member B16	Tumor antigen		-3.24	0.013
ENSECAG00000017619	*NOXO1*	NADPH oxidase organizer 1	Phospholipid binding Superoxide-generating NADPH oxidase activator activity	Extracellular matrix disassembly Positive regulation of cell killing Regulation of hydrogen peroxide metabolic process Superoxide metabolic process Regulation of respiratory burst	-2.67	0.034
ENSECAG00000023392	*ZBTB8A*	zinc finger and BTB domain containing 8A	Metal ion binding DNA binding	Regulation of transcription by RNA polymerase II Transcription regulation	-2.02	5.84E-05
ENSECAG00000019702	*VPS52*	VPS52 subunit of GARP complex	Syntaxin binding	Ectodermal cell differenciation Embryonic ectodermal digestive tract development Endocytic recycling Lysosomal transport Protein transport	-1.26	0.001
ENSECAG00000011960	*DESI1*	desumoylating isopeptidase 1	Hydrolase Identical protein binding	Protein desumoylation Protein export from nucleus Protein modification by small protein removal	-1.23	0.001
ENSECAG00000020433	*TENM3*	teneurin transmembrane protein 3	Cell adhesion molecule binding Protein heterodimerization activity Protein homodimerization activity	Cell adhesion Differenciation Neuron development Signal transduction	-1.00	0.015

ENSECAG00000015867	*PAQR3*	progestin and adipoQ receptor family member 3	Signaling receptor activity	Negative regulation of MAP kinase activity Negative regulation of neuron projection development Negative regulation of protein phosphorylation	-0.84	0.018
ENSECAG00000004931	*MARS2*	methionyl-tRNA synthetase 2, mitochondrial	Aminoacyl-tRNA synthetase Ligase ATP binding	Protein biosynthesis Methionyl-tRNA aminoacylation tRNA aminoacylation for protein translation	-0.82	0.020
ENSECAG00000034815	*VHL*	Von Hippel-Lindau tumor suppressor	Enzyme binding Transcription factor binding	Ubl conjugaison pathway Cell morphogenesis Negative regulation of apoptotic process Negative regulation of cell population proliferation Negative regulation of gene expression Negative regulation of transcription by RNA polymerase II Positive regulation of cell differenciation Positive regulation of transcription Protein stabilization Proteolysis	-0.81	0.018
ENSECAG00000007262	*EEA1*	early endosome antigen 1	GTP-dependant protein binding Protein homodimerization activity	Early endosome to late endosome transport Endocytosis Vesicle fusion	-0.79	0.015
ENSECAG00000009590	*GSTCD*	glutathione S-transferase C-terminal domain containing			-0.72	0.037

ENSECAG00000014243	*GATM*	glycine amidinotransferase	Amidinotransferase activity	Creatine metabolic process Multicellular organism development	0.73	0.018
ENSECAG00000041817	*RBP1*	retinol binding protein 1	Retinoid binding	Lipid homeostasis Retinoic acid biosynthetic process	1.13	0.020
ENSECAG00000010447	*EFEMP1*	EGF containing fibulin extracellular matrix protein 1	Calcium ion binding Epidermal growth factor receptor binding	Embryonic eye morphogenesis Epidermal growth factor receptor signaling pathway Regulation of transcription, DNA-templated	1.95	0.008
ENSECAG00000010385	*MET*	MET proto-oncogene, receptor tyrosine kinase	ATP binding Protein phosphatase binding Transmembrane receptor protein tyrosine kinase activity	Branching morphogenesis of an epithelial tube Cell migration Cell surface receptor signaling pathway MAPK cascade Phagocytosis Positive regulation of microtubule polymerization Positive regulation of transcription by RNA polymerase II	2.83	0.013
ENSECAG00000022277	*MAOA*	Monoamine oxidase A	Monoamine oxidase activity Oxidoreductase	Cellular biogenic amine metabolic process Cytokine-mediated signaling pathway Dopaine catabolic pathway Positive regulation of signal transduction	4.51	0.001

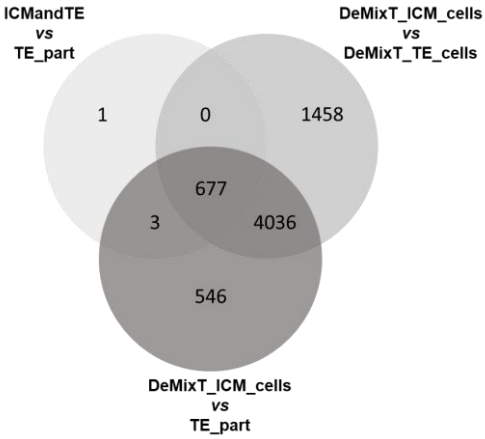
Log2 Fold-Change<0 indicates down-regulation of the gene in embryos from nulliparous mares, also indicated in green; Log2 Fold-Change>0 indicates up-regulation of the gene in embryos from nulliparous mares, also indicated in blue.

Table 4: Up- and down-regulated genes coding for a protein in the trophoblast part of equine embryos according to mare parity

Ensembl Name	Entrez Gene ID	Description	GO Molecular function	GO Biological Process	log2 Fold Change	padj
ENSECAG00000008177	*PRXL2B*	peroxiredoxin like 2B	Antioxydant activity Prostaglandin-F synthase activity	Prostaglandin biosynthetic process	-2.15	0.006
ENSECAG00000012493	*SLC47A1*	solute carrier family 47 member 1	Amide transmembrane transporter activity L-amino acid transmembrane transporter activity	Amino acid import across plasma membrane L-arginine import across plasma membrane Organic cation transport	-1.47	0.001
ENSECAG00000018727	*EFCAB11*	EF-hand calcium binding domain 11	Calcium ion binding		0.51	0.046
ENSECAG00000020415	*FAM162A*	family with sequence similarity 162 member A		Cellular response to hypoxia Positive regulation of apoptotic process Positive regulation of release of cytochrome c from mitochondria	0.65	0.041
ENSECAG00000012554	*GTPBP8*	GTP binding protein 8 (putative)	GTP binding Metal ion binding		0.93	0.044

Log2 Fold-Change<0 indicates down-regulation of the gene in embryos from nulliparous mares, also indicated in green; Log2 Fold-Change>0 indicates up-regulation of the gene in embryos from nulliparous mares, also indicated in blue.

A


ICMandTE
vs
TE_part
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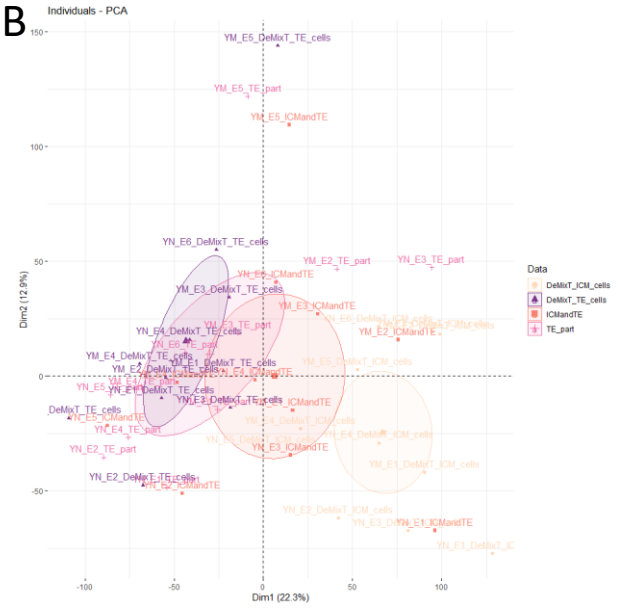
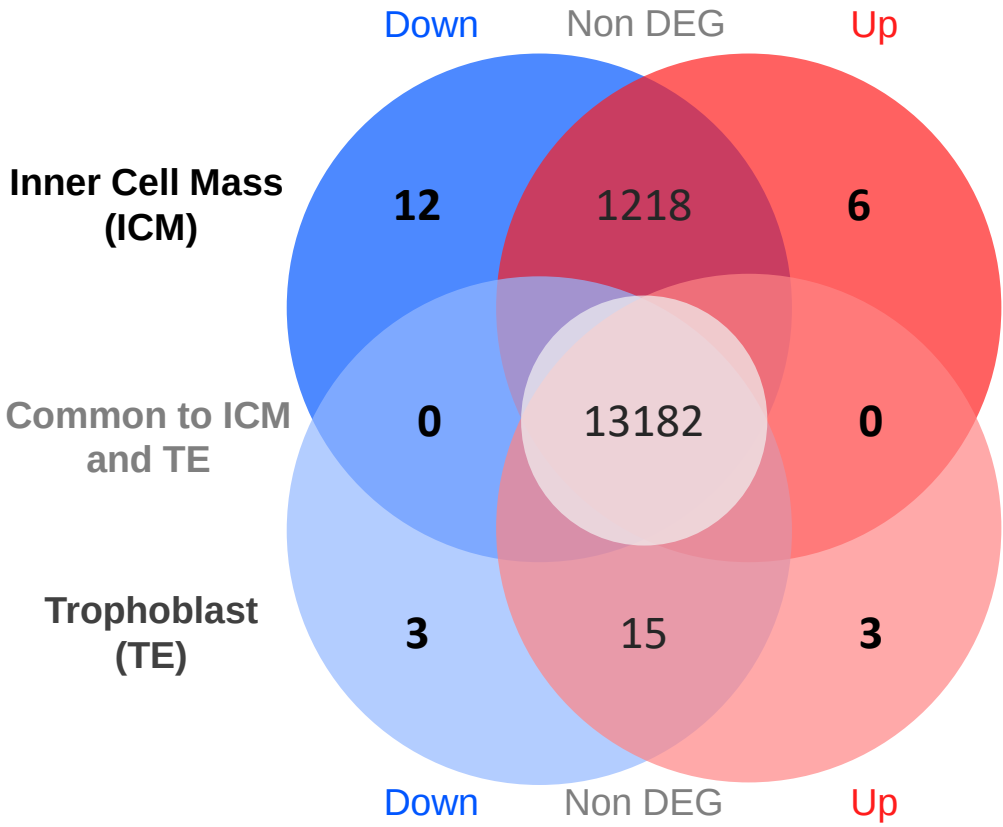

Individuals - PCA
Dim1 (22.3%)
Dim2 (12.9%)
Data
DeMixT_ICM_cells
DeMixT_TE_cells
ICMandTE
TE_part


Down
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Up
Inner Cell Mass (ICM)
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Common to ICM and TE
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Non DEG
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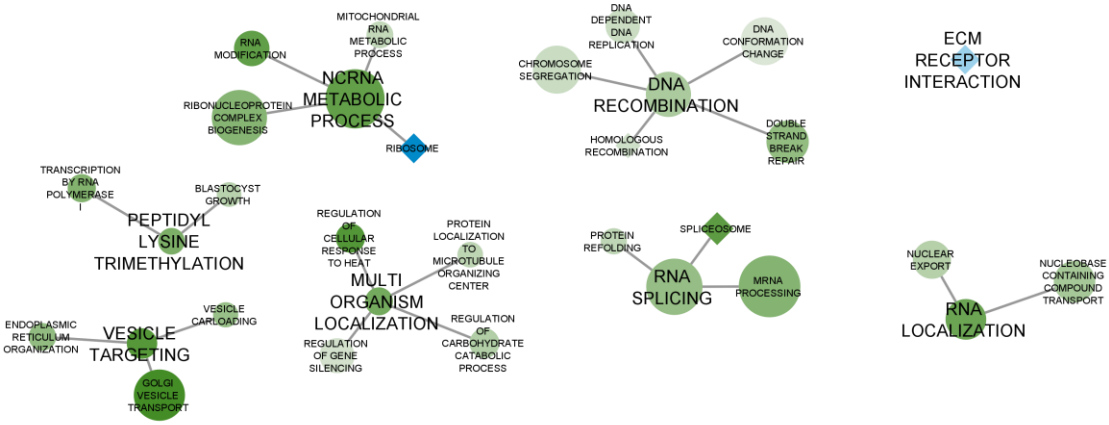
Inner cell mass



Trophoblast