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

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Declarations

Ethics approval and consent to participate

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.

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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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-

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 and paired end, non-oriented RNA sequencing (Illumina, NextSeq500) was performed on each hemi-embryo. To discriminate gene expression in the ICM from that in the TE, deconvolution (DeMixT R package) was used on the ICMandTE dataset. Differential expression was analyzed (DESeq2) with embryo sex and diameter as cofactors using a false discovery rate < 0.05 cutoff. Functional annotation and classification of differentially expressed genes and gene set enrichment analysis were also performed.

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

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

Keywords

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

Background

Unlike other farm animals, the main purpose for the use of horses is athletic performance and leisure. In many cases, mares produce foals regularly to remain profitable after their sport career so that their reproductive career may last until advanced age, even though mares' fertility declines with age [1]. Indeed, in Thoroughbreds, pregnancy rates per estrous at Day 42 post-ovulation were shown to be reduced from 63.0% in young mares to 58.5% in mares older than 9 , and to reach 43.8% in mares older than 18 [2]. One study on 535,746 fertility records in mixed breeds in France showed that if a 10% of fertility decline is chosen as cutoff value for culling breeding mares, then mares older than 8 should not be bred [3].

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

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

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

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

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

Altogether, these data indicate that the quality of embryos from older mares is reduced. Subsequently, dam age could also affect placental and fetal intrauterine and offspring post-natal growth and long-term health, in the context of the Developmental Origins of Health and Diseases (DOHaD) [17]. To our knowledge, the role of the embryo *per se*, however, has not yet been explored. Moreover, age and parity are generally positively correlated and their respective impact have not been determined yet. The aim of this study was to determine the effect of maternal age in the mare on embryo gene expression at the blastocyst stage. Young (<10 years) and older (>10 years) multiparous mares were inseminated with the same stallion. Blastocysts were collected 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. Deconvolution was used to isolate gene expression of inner cell mass from trophoblast cells in ICMandTE part. Results shows that maternal age alters gene expression of both equine Day 8 trophoblast and inner cell mass.

Materials and methods

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.

Embryo collection

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

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

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

All mares were multiparous.

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

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

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 [19,20]. At the same time, mares were inseminated once with fresh or refrigerated 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 luproliol an analogue of prostaglandin F2α (i.m; 7.5 mg; Prosolvin, Virbac, France).

Embryo bisection and RNA extraction

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

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

RNA sequencing

Five nanograms of total RNA were mixed (Additional file 1) with ERCC spike-in mix (ThermoFisher Scientific, France) according to manufacturer 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). Libraries 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) (Additional file 1). Demultiplexing was performed with bcl2fastq2 version 2.2.18.12 (Illumina, France) and adapters were trimmed with Cutadapt version 1.15 [21]. Only reads longer than 10pb were kept.

Embryo sexing

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

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

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

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

RNA mapping and counting

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

Data analysis

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

Embryo recovery rate, embryo diameter and total RNA content analysis

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

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

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

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

Deconvolution of gene expression in ICMandTE using DeMixT

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

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

Quality checks for deconvolution

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

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

Maternal age comparison for gene expression

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

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

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

Gene set enrichment analyses (GSEA)

The count values were log transformed 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) [41,42] to identify biological gene sets disturbed by age. GSEA was performed using the following parameters: 1000 gene set permutations, weighted enrichment analysis, gene set size between 15 and 500, signal-to-noise metrics. Molecular Signatures Database [43] version 7.1 (C2:

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

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

Results

Embryo rates, diameter, total RNA content and quality

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

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

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

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

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

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

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

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

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

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

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

Gene expressions were obtained from RNA of 11 equine embryos bisected in two hemi-embryos: one part is composed only of trophoblast (TE), TE_part, while the other part is composed of TE and inner cell mass (ICM), ICMandTE. As it is impossible to estimate the proportion of each cells in ICMandTE, deconvolution algorithm (package DeMixT) was used to estimate gene expression of these different kind of cells. DeMixT_ICM_cells dataset is 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, gene is more expressed in ICMandTE or DeMixT_ICM_cells while when log2FC < 0, gene is more expressed in TE_part. Green is used to represent gene differentially expressed in the present study. Orange is used to represent gens that have been previously identified as predominant in the ICM [23] but which are identified here as predominant in the TE.

For further analysis, DeMixT_ICM_cells and TE_part gene expression datasets are presented. Nevertheless, the comparison of maternal age on ICMandTE dataset (graphics, differential gene expression analysis and GSEA) was also performed and this analysis is available in Additional file 2, 3 and 4.

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

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

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

Differential gene expression in pure TE part gene expression

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

Gene set enrichment analysis in deconvoluted ICM cells' genes expression according to maternal age

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

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

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

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

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

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

Gene set enrichment analysis in TE

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

0.05 and $\log_2FC = -0.74$, $FDR < 0.05$) in OM ICM. *ARHGAP35* codes for P190 Rho GTPase activating protein that mediates signalization through integrin-dependent adhesion in cells. Aberrant neural morphogenesis, related to an accumulation of polymerized actin in embryoneural tube cells, is reported in mice lacking P190 Rho GTPase activating proteins [64]. Furthermore, P21 activated kinases (PAK) are effector kinases for other small Rho GTPases such as Rac and regulate the polymerization of actin and can affect the microtubule organization and therefore the regulation of cytoskeleton [65]. Once activated by this signaling pathway, PAKs promote the turnover of focal adhesions. PAKs will then, trigger p38 complex activation that regulates transcription [66]. *PAK3* was downregulated in the OM ICM ($\log_2FC = -3.76$, $FDR < 0.001$). However, in TE, *SLIT-ROBO Rho GTPase Activating Protein 3*, *SRGAP3* was one of the upregulated genes in OM ($\log_2FC = 2.52$, $FDR < 0.01$). This gene regulates cytoskeletal reorganization and plays an important role in the formation of cell-cell adhesion [67]. Therefore, maternal aging seems to alter focal adhesion in both ICM and TE of embryos but, while in ICM, it seems to reduce cohesion and signalization, in TE it seems to reinforce them.

Several signaling pathways required for development were altered and numerous genes related to focal adhesions and signaling pathways were down-regulated in OM ICM (*ARHGAP9* and *ARHGAP35*; *B-Raf Proto-Oncogene, Serine/Threonine Kinase, BRAF*; *Filamin C, FLNC*; *FYN Proto-Oncogene, Src Family Tyrosine Kinase, FYN*; *PAK3*; *Protein Phosphatase 1 Regulatory Subunit 12A, PPP1R12A*; *Talin 2, TLN2*; *X-Linked Inhibitor Of Apoptosis, XIAP*). Among all signaling pathways, transforming growth factor beta ($TGF-\beta$), Wg et Int (Wnt), mitogen-activated protein kinases (MAPK) and apoptosis signaling were altered by maternal age in equine embryos. Indeed, *transforming growth factor beta receptor 1 (TGFB1)* was downregulated ($\log_2FC = -4.34$; $FDR < 0.05$) in the ICM of embryos from old mares. *TGFB1* is one of the two dimers for the receptor for transforming growth factors beta. This factor family is essential in the generation of axes and cell fate commitment during mammal embryogenesis [68]. Therefore, the reduced production of $TGF-\beta$ receptor in ICM could impair the establishment of cell lines in old mares' embryos. Moreover, *Casein Kinase 1 Alpha 1, (CSNK1A1)* and *Protein Phosphatase 2 Regulatory Subunit B'Epsilon (PPP2R5E)*, involved in Wnt signaling pathway, were

downregulated in OM embryos. Wnt signaling pathway is also involved in nervous development system and may act on cell determination in mice embryos [69]. In horses, gastrulation begins by Day 12 post ovulation and the primitive streak develops quickly after mesoderm formation [70]. It is therefore possible that the alteration of the Wnt signaling pathway could have a negative impact on the brain development of the future fetus. This hypothesis could be confirmed by the fact that *DMRT3* (*Doublesex And Mab-3 Related Transcription Factor 3*) was downregulated in the OM ICM ($\log_2FC = -3.07$, $FDR < 0.05$). *DMRT3* is required for the proper development of the brain in prenatal chicken and mice [71]. Moreover, mutations of this gene are responsible for different gaits in horses (specific to aptitude to trot instead of cantering) [72]. Therefore, altogether, the data indicate that cell fate commitment in ICM seems to be altered by maternal aging.

Cell cycle related pathways and genes are also disturbed by maternal aging. Indeed, the expression of *Pleckstrin Homology domain-Interacting Protein (PHIP)*, also known as replication initiation determinant, was reduced ($\log_2FC = -0.86$, $FDR < 0.01$) in the OM ICM. This gene is required for the initiation of DNA replication. Fibroblasts originated from mice embryos with depleted *PHIP* expression presented less replication-initiation as well as abnormal replication fork progression events [73]. Moreover, several cyclins (CCN) were less expressed in the ICM of embryos from old mares (*CCNB3*, *CCNI* and *CCNT2*). Cyclins are key cell cycle regulators and, for instance cyclin B3 degradation allows the passage from metaphase (chromatids aligned on metaphase plate) to anaphase (segregation of sister chromatids) [74]. During metaphase and anaphase, spindle assembly and chromosome segregation could also be compromised. Indeed, genes involved in the formation of microtubules (*Tubulin Gamma 2*, *TUBG2*) [75], the generation of the mitotic spindle (*Never in Mitosis A related kinase 9*, *NEK9*) [76], the production of cohesion and condensing complexes (*Structural Maintenance Of Chromosomes 4*, *SMC4*) [77], the control of centriole length, important for centrosomes and microtubules organization (*Centriolar Coiled-Coil Protein 110*, *CCP110*) [78], the amplification of intra-spindle microtubules allowing the formation of a sufficient quantity of kinetochore (*HAUS Augmin Like Complex Subunit 6*, *HAUS6*) [79], the stabilization and protection of the cohesin complex association

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

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

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

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

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

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

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

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

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

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

Conclusion

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

List of abbreviations

ARHGAP35: Rho GTPase Activating Protein 35

ARHGAP9: Rho GTPase Activating Protein 9

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	CDC45: 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- β : 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

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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.

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

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

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

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

Node shape represents the gene set database: GO BP (circle) or KEGG (diamond). Blue nodes represent enriched gene sets in YM (NES < 0) while red nodes represent enriched gene sets in OM (NES > 0).

Edges represent the level of connection between representative gene sets. This graph was performed using SUMER R package [44] and modified using cytoscape 3.8.2 [45]

ICM: Inner Cell Mass; TE: trophoblast; FDR: False Discovery Rate; GO BP: Gene Ontology Biological Process; Kyoto Encyclopedia of Genes and Genomes; NES: Normalized Enrichment Score; YM: Young mares; OM: Old mares

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

Normal blastocyst development from day 7 to day 9 is represented inside black square using literature [56,57]. Light blue boxes represent biological processes that are enriched in YM embryos and light red boxes represent biological processes that are enriched in OM embryos.

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:

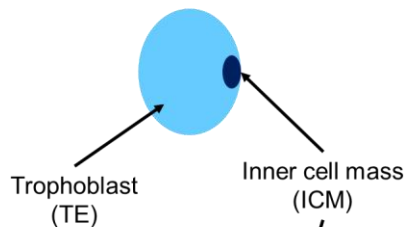
1165 Additional file 7.csv

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.

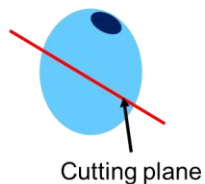
A)



Trophoblast
(TE)

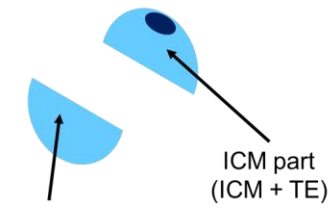
Inner cell mass
(ICM)

B)



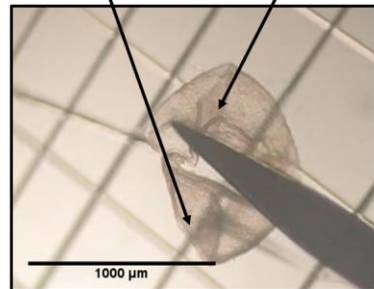
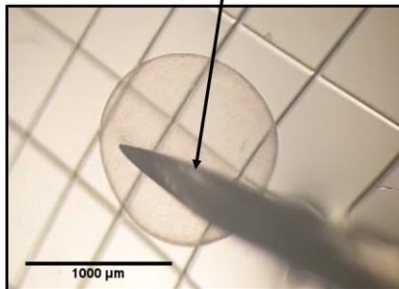
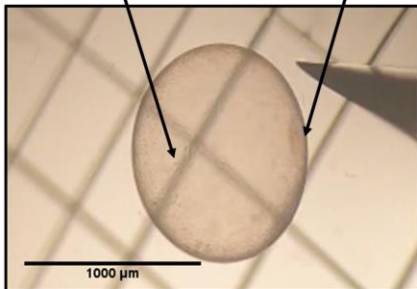
Cutting plane

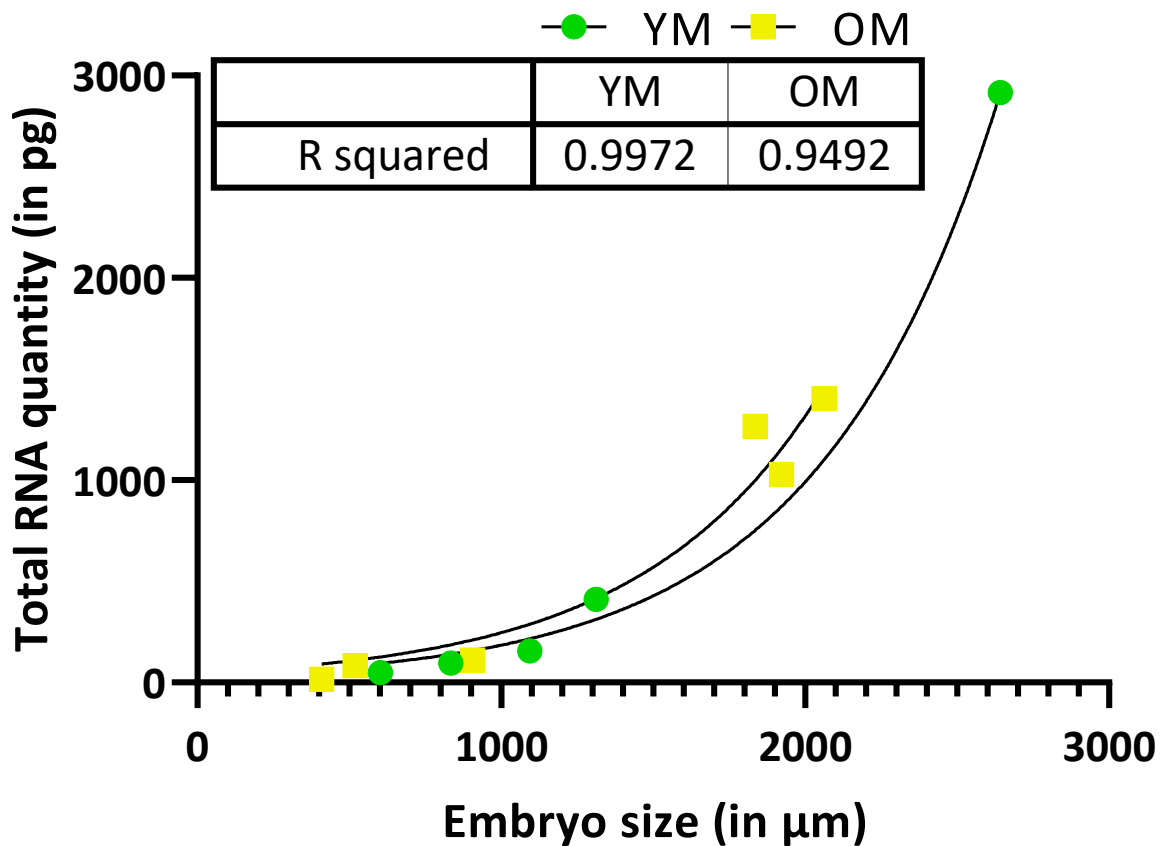
C)



TE part

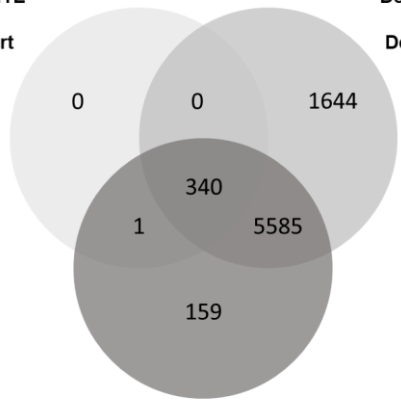
ICM part
(ICM + TE)





A

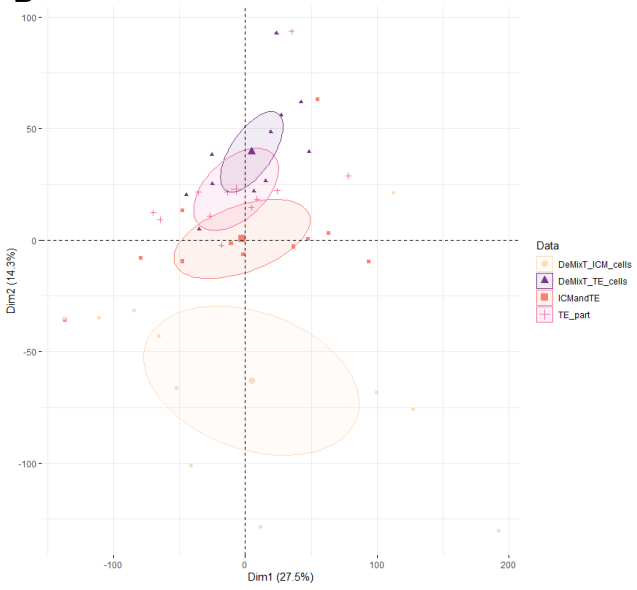
ICMandTE
vs
TE_part



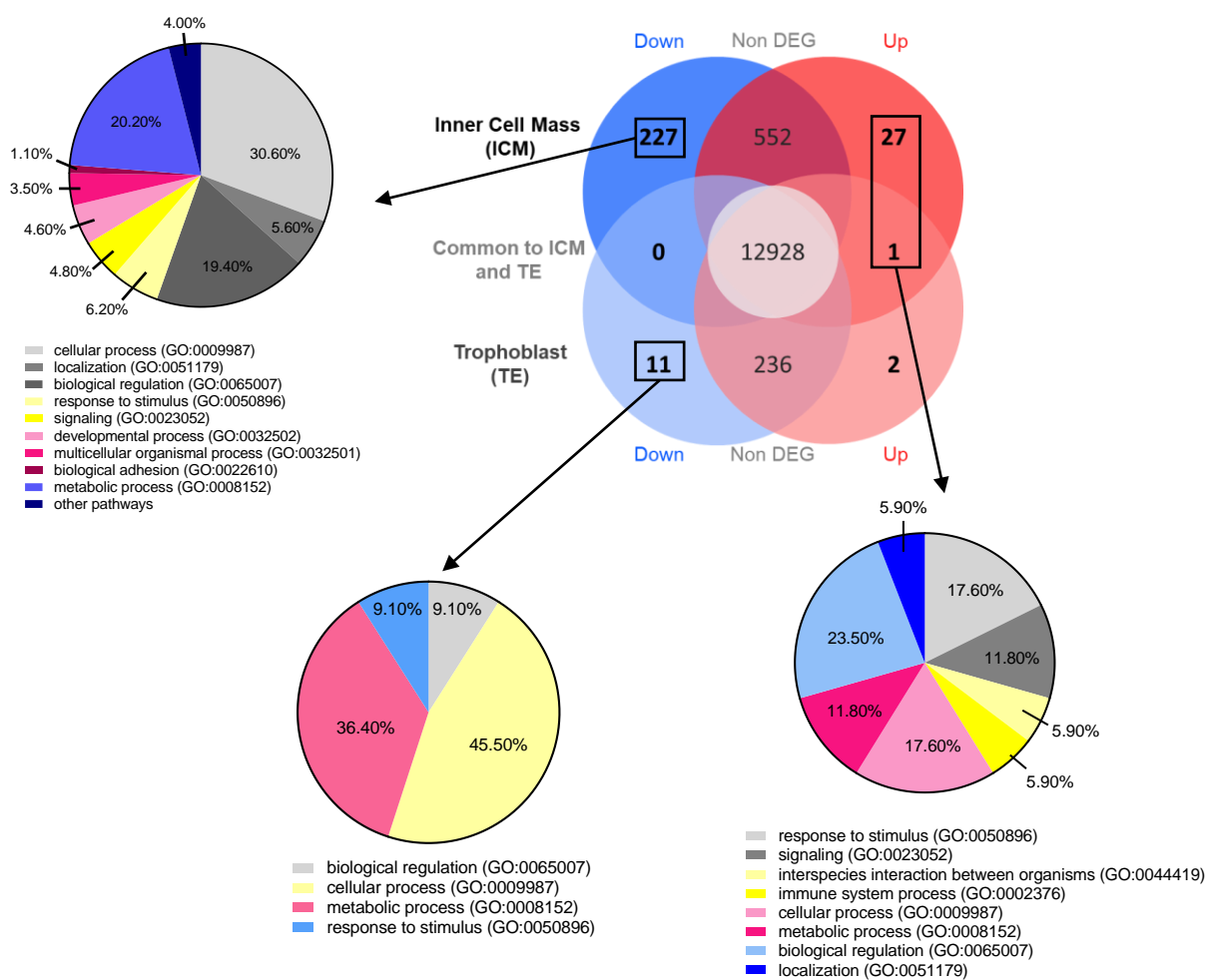
DeMixT_ICM_cells
vs
TE_part

B

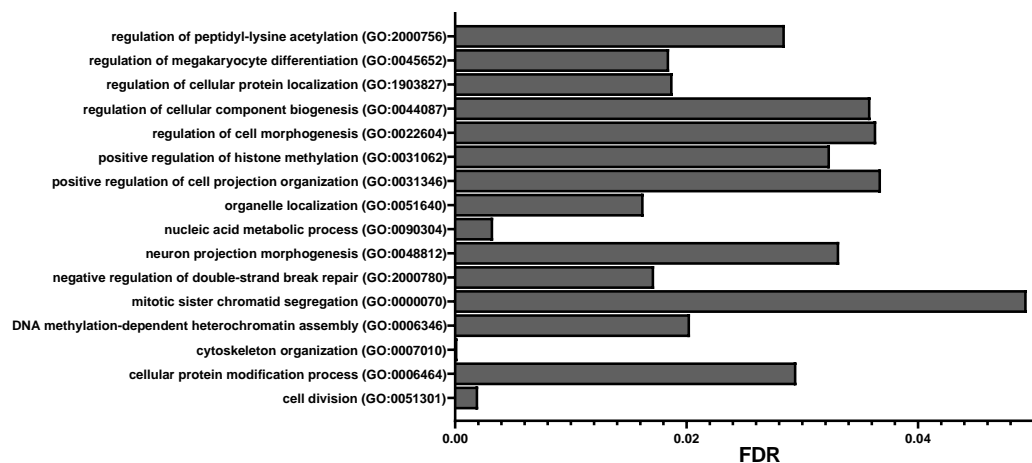
Individuals - PCA



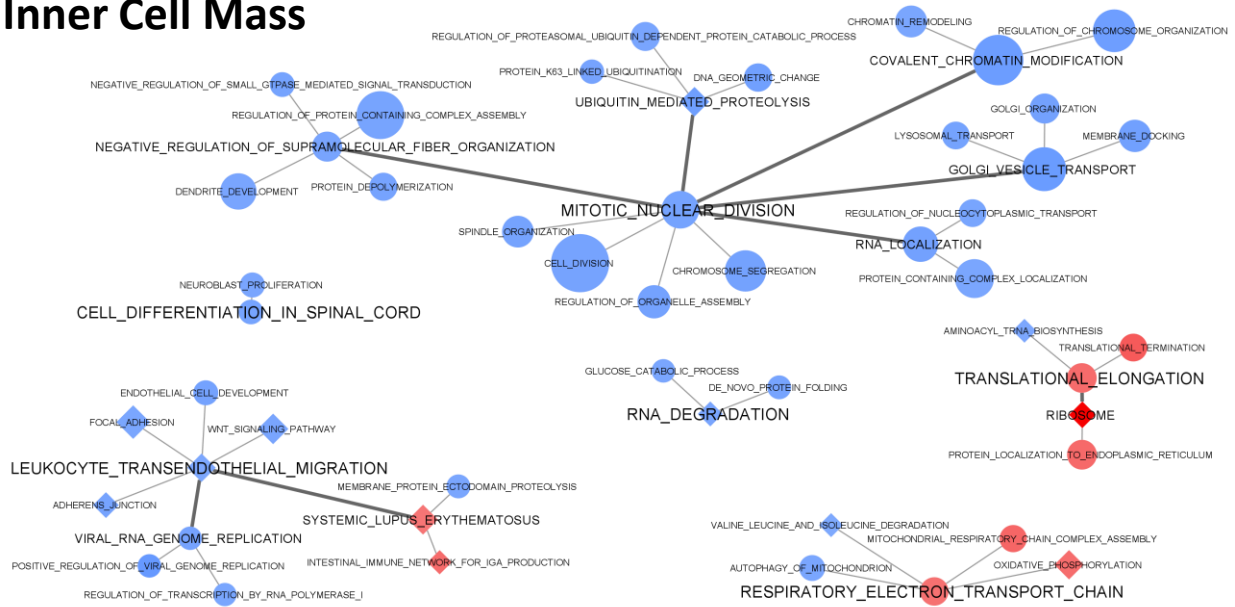
A



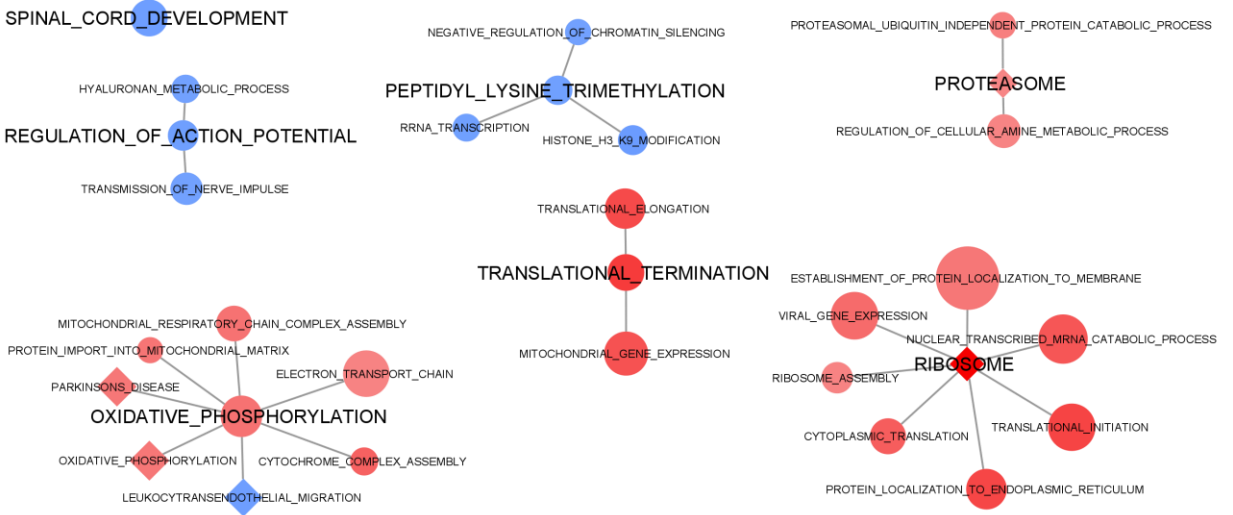
B



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

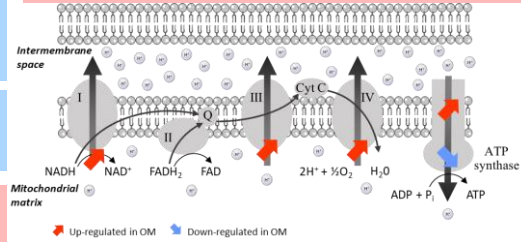
Embryo development

- Cell fate commitment

Translation

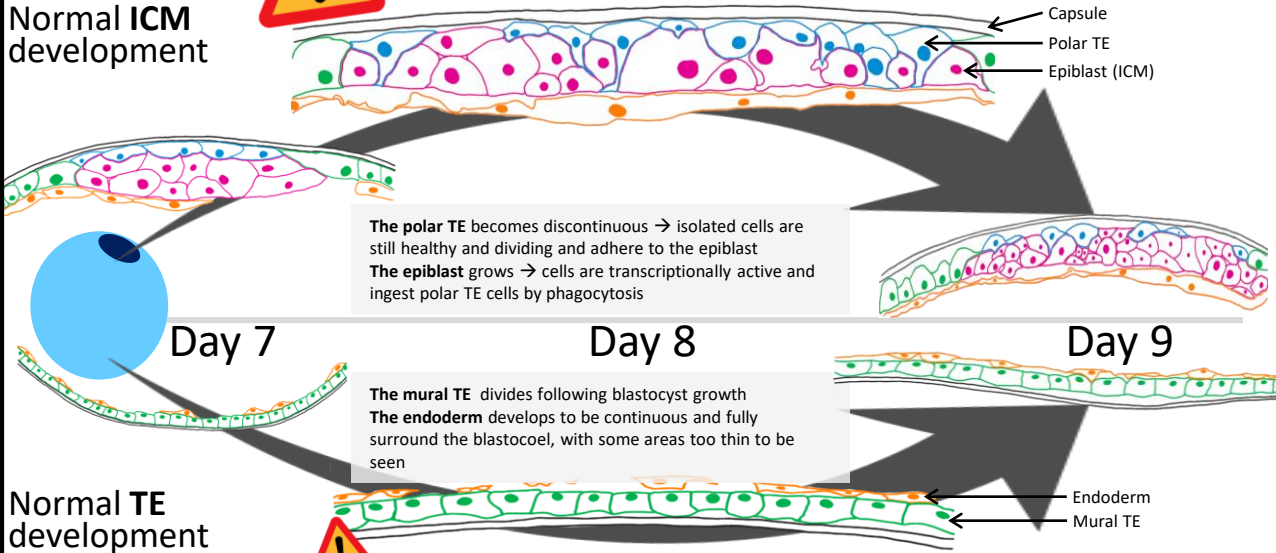
- Ribosome biogenesis

Mitochondrial function



ALTERED ICM DEVELOPMENT

Normal ICM development



ALTERED TE DEVELOPMENT

Ion movement

- Transmission of action potential

Transcription

- Chromatin modification

Embryo development

Protein catabolism

- Proteasome

Mitochondrial function

Translation

- Ribosome biogenesis

MATERNAL AGING