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Comparative transcriptome analysis of embryo invasion in the mink uterus

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A B S T R A C T

Introduction: In mink, as many as 65% of embryos die during gestation. The causes and the mechanisms of embryonic mortality remain unclear. The purpose of our study was to examine global gene expression changes during embryo invasion in mink, and thereby to identify potential signaling pathways involved in implantation failure and early pregnancy loss.
Methods: Illumina's next-generation sequencing technology (RNA-Seq) was used to analyze the differentially expressed genes (DEGs) in implantation (IMs) and inter-implantation sites (inter-IMs) of uterine tissue.
Results: We identified a total of 606 DEGs, including 420 up- and 186 down-regulated genes in IMs compared to inter-IMs. Gene annotation analysis indicated multiple biological pathways to be significantly enriched for DEGs, including immune response, ECM complex, cytokine activity, chemokine activity and protein binding. The KEGG pathway including cytokine-cytokine receptor interaction, Jak-STAT, TNF and the chemokine signaling pathway were the most enriched. A gene network was constructed, and hub nodes such as CSF3, ICAM1, FOS, IL1B, IL8, CD14 and MYC were found through network analysis.
Discussion: This report provides a valuable resource for understanding the mechanisms of embryo implantation in mink.

1. Introduction

Implantation in mustelid carnivores has several unique characteristics. Trophoblastic cells do not extrude through the zona pellucida of expanded blastocysts just prior to attachment of the embryo [1]. Mink also exhibit a high degree of trophoblast invasion of the uterine epithelium at the site of embryo attachment, but although the maternal blood vessels are surrounded by trophoblast cells, the integrity of the vessels is maintained [2], The resultant placenta is endotheliochorial and decidual, and is described as a discontinuous zonary type [3]. Embryo growth and survival are dependent upon establishment of the placenta, which in turn guarantees a nutrient supply, gas exchange and waste removal. Defective trophoblastic invasion is associated with abnormalities of placental development and resulting fetal growth defects have been reported in primary [4], mouse [5], bovine [6] and sheep [7].

Prenatal mortality is a critical barrier to successful mink reproduction. Approximately 60% of embryos are lost during pregnancy, and about 20% of these are lost during the pre-implantation period [8]. The reasons and mechanisms for embryonic mortality in mink are not well understood. The sparse research on mink reproduction mainly focuses on embryo diapause and its regulation. The molecular pathways involved in this process include prolactin-induced ODC1 expression in the uterus via pSTAT1 and mTOR, thereby regulating uterine polyamine levels [9], important genes include COX2, LIF [10], VEGF [11] and Msx [12].

Trophoblast cell differentiation, invasion and placental formation in mink has received little attention. Desmarais [13] reported that nuclear receptors of the peroxisome proliferator-activated receptor (PPAR) family are implicated in implantation and early placental formation in mink. Lopes [14] reported that prostaglandin E (PGE) regulates uterine and placental vascular development through activation of VEGF transcriptional activity. So far, genuine candidate genes and molecular mechanisms involved in embryo invasion in mink remain largely unknown. In recent years, high-throughput transcriptomic approaches have been developed, making it possible to study genome-wide gene expression and complex signaling networks. By using these approaches, several studies have reported altered uterine gene expression profiles associated with embryo implantation in humans [15], mice [16], sheep [17], cows [18] and pigs [19].

In this study, we present the first application of RNA-Seq in mink by sequencing RNA from the uterus in IMs and inter-IMs. The data from

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this study provide information about genes involved in early embryonic invasion in the mink uterus. Our study contributes toward a deeper understanding of the molecular events associated with implantation failure and early pregnancy loss in mink.

2. Materials and methods

2.1. Animals and sample collection

All procedures involving animal care were approved by the experimental animal use and care committee of the Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences (ISAPSWAPS2016000302). All females were mated with two fertile males, at one week intervals, according to the usual commercial farming procedures. A successful mating was confirmed by the presence of motile spermatozoa in vaginal smears. In mink, termination of embryonic diapause is induced by increasing secretion of prolactin from the pituitary gland [20–22], which stimulates luteal function thereby increasing progesterone secretion associated with blastocyst reactivation and implantation. A previous study showed that diapause can be artificially terminated by treatment with prolactin [21], Our study used a standard protocol consisting of injecting 1 mg/kg/d of ovine prolactin (Sigma) daily beginning 7–9 days following the last mating and continuing for 12 days. We collected uterine samples on day 13 after the initiation of Prolactin injection, at the time when implantation occurs, as previously described [21,22]. IMs were identified by injection of 0.1 ml of 1% Chicago blue (Sigma) in 0.85% sodium chloride. The IMs and inter-IMs from mink uterus were collected and stored in liquid nitrogen for RNA-Seq.

2.2. RNA-seq analysis

Total RNA was extracted from uterus samples using TRIzol® reagent (Invitrogen, n = 3 for each IMs and inter-IMs group). RNA integrity was assessed by the Agilent Bioanalyzer 2100 system. The following RNA quality control parameters were used: A260/A280 ratio ≥1.8, A260/A230 ratio ≥2.0 and RIN (RNA integrity number) value ≥ 7.0.1.5 μg RNA from each sample was used as input material for the RNA samples preparations. Sequencing libraries were generated using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina (NEB, USA) and index codes were added to attribute sequences to each sample. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina). After cluster generation, the libraries were sequenced on an Illumina Hiseq platform and paired-end reads were acquired. Raw reads were firstly preprocessed and filtered to clean reads through in-house perl scripts. Transcriptome assembly was based on the left.fq and right.fq using Trinity [23] with min_kmer_cov set to 2 by default and all other parameters set to default. Gene function was annotated from the National Center for Biotechnology Information (NCBI). Differential expression analysis of two groups was performed by using the DESeq R package. The P-values and false discovery rate were adjusted and controlled by using the Benjamini and Hochberg approach. The DEGs were screened based on DESeq analysis [24], taking $|\log2(\text{fold change})| > 1$ and $p < 0.05$ as the cut-off.

2.3. Quantitative real-time PCR validation of mRNA

RNA samples were isolated from an independent set of biological replicates from those used in the RNAseq (n = 3 for each IMs and inter-IMs group). Total RNA was extracted from IMs and inter-IMs of uterine samples with Trizol® Reagent (Invitrogen). RNA samples were treated with DNaseI (invitrogen) to eliminate potential genomic DNA contamination. RNA integrity was assessed by the Agilent Bioanalyzer 2100 system. The quality and quantity of the RNA were verified by optical density reading using a NanoDrop 200c spectrophotometer (Thermo Scientific). Only RNA samples meeting the following requirements were used in this study, the ratios of A260/280 was between 1.8 and 2.2, A260/A230 ratio no less than 2, the ratio of 28S/18S ribosomal RNA should be between 1.5 and 2.0, electrophoresis bands on agarose gel were clear, and RIN (RNA integrity number) value ≥ 7.0. One μg of total RNA was used for cDNA synthesis in a 20 μl transcript system, including 4 μl TransScript® All-in-One SuperMix, 1 μl gDNA Remover, and variable RNase-Free Water. Real-time PCR was determined using a CFX96 Real-time System (Bio-Rad) with 20 μl PCR reaction mixture that included 10 μl 2 × SG Fast qPCR Master Mix (BBI Life Sciences), 0.4 μl of 10 μM forward primer, 0.4 μl of 10 μM reverse primer, 2 μl DNF buffer, 0.5 μl of cDNA and 6.7 μl PCR-grade water. The reaction conditions were 95 °C for 3 min, then 40 cycles of 95 °C for 3s, 60 °C for 30s, and 72 °C for 20s. GAPDH was used as a reference gene for normalization, which was previously used in studies on the mink endometrium [11–14]. RT negative control reactions contained RNase-Free Water instead of total RNA. Non-template control reactions contained nuclease-free water instead of templates, and plates were discarded if more than one of the negative control triplicates was contaminated. Individual samples of a triplicate were discarded if they had irregular melting curves or if the coefficient of variation was greater than 0.02. The qRT-PCR was perfomed in three biological and three technical replicates. Relative expression levels of the selected genes were calculated using the CFX Manager software. Statistical analyses of qRT-PCR data were performed using Student's paired *t*-test, and differences with $p < 0.05$ were considered to be significant. Primers for each selected gene were designed using primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primer-blast/.) and are listed in Supplementary materials 6 (Table S2).

2.4. Gene ontology (GO) pathway and network analysis

GO enrichment analysis of DEGs was implemented using the GOseq R packages based on Wallenius non-central hyper-geometric distribution [25], which can adjust for gene length bias in DEGs. The significance cutoff for adjusted p-values (Benjamini-Hochberg multiple test correction method) was set at 0.05. KOBAS software was used to test the statistical enrichment of DEGs in the KEGG pathway. The sequences of DEGs were blast (blastx) to the genome of a related species (http://string-db.org/) to obtain the predicted PPI (protein protein interaction) of these DEGs. Then the PPI of these DEGs were visualized in Cytoscape. In the PPI network, nodes stand for proteins, and edges represent interactions between 2 proteins.

3. Results

3.1. Identification of transcriptomic differences

RNA-Seq libraries were prepared for IMs (n = 3) and inter-IMs (n = 3) of mink uterus. Data analysis revealed that sequencing of the libraries yielded more than 55.7 and 57.4 million reads for the IMs and inter-IMs groups, respectively. After removing invalid reads, we acquired more than 53.6 and 55.0 million clean reads from each group. A total of 160,816 unigenes were obtained from clean reads using the optimized parameters.

We identified a total of 606 DEGs. Among them, 420 genes were up-regulated and 186 genes were down-regulated at IMs in comparison to inter-IMs. Of these, 582 annotated genes (376 genes fold > 2, and 97 genes fold > 1.5) were differentially expressed between IMs and inter-IMs, 402 annotated genes (223 genes fold > 2, and 84 genes fold > 1.5) were upregulated, and 180 annotated genes (153 genes fold > 2, and 13 genes fold > 1.5) were down-regulated at IMs in comparison to inter-IMs (Table 1). Annotated DEGs are shown in Supplementary materials l (Table S1). The top 20 most DEGs (highest P value) are presented in Table 2.

Table 1
Number of DEGs between IMs and inter-IMs.

comparison	Annotated DEGs (All DEGs) with fold change of 2 or greater.	Annotated DEGs (All DEGs) with fold change greater than 1.5 and less than 2	All annotated DEGs (All DEGs)
IMs > Inter-IMs	223 (245)	84(84)	402 (420)
Inter-IMs > IMs	153 (159)	13 (13)	180 (186)
All	376 (404)	97 (97)	582 (606)

Statistical significance was reached at $P < 0.05$ for all genes.

3.2. GO enrichment analysis

Enriched GO terms are classified according to biological process (BP), molecular functions (MF) and cellular components (CC). In the BP category, 400 terms were significantly enriched, including immune response, chemotaxis, signal transduction, cell adhesion, cell migration, and regulation of inflammatory response. The enriched CC categories were: extracellular matrix, basement membrane, cell projection, and plasma membrane receptor complex. With respect MF, protein binding, cytokine activity, chemokine activity and metallopeptidase activity were significantly enriched (Table 3). Among these top GO terms, cell adhesion from BP, extracellular matrix from CC and protein binding from MF were all involved in cell adhesion (Supplementary materials 2, Figure S1), indicating their important roles during embryo invasion in mink. Genes such as CSF3, IL (IL1B, IL6, IL8, IL3RA), CCL (CCL13, CCL15, CCL8), CXCL (CXCL14, CXCL1, CXCL8), LAMA3 and LIF were more highly expressed in IMs compared to inter-IMs.

3.3. KEGG pathways analysis of DEGs

KEGG pathway enrichment analysis was done for DEGs and $P < 0.05$ was used as a cutoff. A total of 214 enriched pathways were found between the IMs and inter-IMs groups. The classifications indicated that cytokine-cytokine receptor interaction (Supplementary materials 3, Figure S2), Jak-STAT signaling pathway (Supplementary materials 4, Figure S3), TNF signaling pathway (Supplementary materials 5, Figure S4) and chemokine signaling pathway were highly enriched in IMs comprared to inter-IMs. The top 10 KEGG pathways are shown in Table 4.

3.4. qRT-PCR validation of RNA-Seq

To validate the RNA-Seq results, we selected the top DEGs and the genes in the top GO/KEGG pathway, which may play an important role during embryo invasion in mink. The expression trends of these genes determined by qRT-PCR showed good agreement with the RNA-seq results (Fig. 1), all of them reaching the significance level of $P < 0.05$.

3.5. PPI network construction

We used STRING to obtain PPI relationships. The nodes of degree ≥ 5 were selected, and finally we obtained a gene network consisting of 120 nodes and 324 edges (Supplementary materials 6, Figure S5). The genes CSF3 (colony stimulating factor 3), ICAM1 (intercellular adhesion molecule 1), FOS (proto-oncogene protein c-fos), IL1B (interleukin 1, beta), IL8 (interleukin 8), CD14 (monocyte differentiation antigen CD14) and MYC (myc proto-oncogene protein) with high connectivity degree were selected as the hub nodes and thus might play important roles in embryo invasion in mink (Fig. 2).

4. Discussion

In this study, we report application of RNA-Seq to identify mechanisms and pathways while the embryo is invading into the endometrium in mink. 606 DEGs were identified at the IMs compared to the inter-IMs ($P < 0.05$). Among these DEGs, we validated 18 genes using qRT-PCR. The expression trend of these genes determined by qRT-PCR showed a good agreement with RNA-seq results, suggesting a high quality of our results. To further understand gene functions and their regulatory mechanisms, GO enrichment and KEGG pathway analysis were performed. The main network showed several genes associated with immune response, cell adhesion and locomotion, cytokines and chemokines activity, ECM complex and protein binding.

4.1. Genes related to immune response

The functional category of immune response was highly over-represented in IMs compared to inter-IMs of the uterus (Table 2). This is in agreement with many previous reports that have found the presence of immune cells at the IMs and establishment of a favorable environment, including the production of cytokines and the expression of adhesion molecules, regulating the invasion of fetal cytotrophoblast cells [26]. Studies with murine models lacking natural killer (NK) cells suggests important roles for immune response in spiral arteriole remodeling and decidualization [27]. Aborted placentas expressed fewer immune-related genes than successful placentas [28]. Several transcriptome analyses in other animals have shown that immune response is among the top ranked enriched terms in IMs compared to inter-IMs [16,19], highlighting the importance of immune response during embryo implantation.

In the present study, we observed 18 DEGs associated with immune response. Upregulated genes including the interleukin family (IL6, IL1B), chemokine (C-C motif) ligand (CCL15, CCL13, CCL8), chemokine (C-X-C motif) ligand (CXCL14, CXCL1, CXCL8), CD molecular (CD1), Septin 9 (SEPT9), HOP homeobox (HOPX), Colony stimulating factor 3 (CSF3), Leukemia inhibitory factor (LIF) and Apelin (APLN). Negatively regulated immune system genes included WDR78, NFKBID, IL3RA and TTC3. LIF is known as a candidate gene regulating litter size in pigs [29]. A study in rhesus monkeys indicated that uterine administration of anti-LIF mAb leads to reduced pregnancy rates [30], while mutations in the LIF gene in humans cause reduced fertility [31], LIF may function by controlling the proportions and numbers of immune cells in the endometrium at the time of implantation. Knocking out the LIF gene reduced the number of endometrial macrophages, and increased the number of NK cells and ecosinophils [32].

Chemokines are a group of small cytokines that are expressed on both sides of the foeto-maternal interface, they can increase leukocyte adhesion to the endothelium through the up-regulation of adhesion molecules and hence promote leukocyte extravasation. In our study, chemokines were up-regulated in IMs compared to inter-IMs in agreement with studies using murine models [16]. Prior studies in human have shown that CXCL8, CXCL1 and IL6 induce trophoblast migration and invasion into deciduas [33,34], so downregulation of these cytokines in the placenta may be responsible for fetal loss and fetal growth retardation. In contrast to other studies in mice and marsupials, we did not see a change in gene expression for immunoglobulins or the CD molecule [35–37], even though these genes are expressed in other tissues in mink.

Table 2
The top 20 most DEGs in IMs compared with inter-IMs ($P < 0.05$).

Gene symbol	log2Fold Change	P value	Gene description	Predicted molecular function
ADAMTSL3	5.751	7.14E-30	ADAMTS-like 3	protein binding
ARKH	5.9045	8.45E-30	ankyrin repeat and KH domain-containing protein 1	proton symporter activity, protein binding, RNA binding
GAS8	−5.4561	1.21E-26	growth arrest-specific 8	cell motility, pathogenesis
LIF	3.5088	6.95E-24	leukemia inhibitory factor	cytokine activity
CSF3	4.0831	3.05E-22	colony stimulating factor 3	interleukin-6 receptor binding, cytokine activity
CFTR	3.2466	2.35E-20	cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)	enzyme binding, nucleotide binding, coupled to transmembrane movement of substances
IL17R	4.8518	5.90E-20	interleukin 17 receptor A	calcium ion binding
FMR	4.668	2.92E-18	fragile X mental retardation 1	RNA binding
BAP1	4.2088	2.99E-14	associated protein-1 (ubiquitin carboxy-terminal hydrolase)	ubiquitin-specific protease activity
POT1	4.4966	1.05E-16	protection of telomeres 1	nucleic acid binding
MTCL1	4.5113	7.43E-16	microtubule crosslinking factor 1	ATP binding, serine/threonine kinase activity, transcription factor activity, sequence-specific DNA binding
RALGAPA2	−4.4638	9.87E-16	Ral GTPase activating protein, alpha subunit 2 (catalytic)	calmodulin binding, spectrin binding, GTPase activator activity
FMR	4.668	2.92E-18	fragile X mental retardation 1	metalloendopeptidase activity
DYRK1	4.2809	8.34E-15	*Equus caballus* dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A	ATP binding//protein kinase activity
LSG1	4.3492	9.44E-15	large 60S subunit nuclear export GTPase 1	ferrous iron transmembrane transporter activity//GTP binding//GTPase activity//zinc ion binding
BAP1	−4.6021	8.57E-18	BRCA1 associated protein-1 (ubiquitin carboxy-terminal hydrolase)	ubiquitin-specific protease activity
SSH	−4.1713	3.92E-14	slingshot protein phosphatase 2	DNA binding, structural molecule activity, protein tyrosine/serine/threonine phosphatase activity
CNOT1	−4.23	4.10E-14	CCR4-NOT transcription complex, subunit 1	protein binding, calcium ion binding
PLCH	−3.9128	4.95E-14	phospholipase C, eta 1	protein binding, calcium ion binding, RNA binding, phosphatidylinositol phospholipase C activity
PTPRR	4.2533	5.94E-14	protein tyrosine phosphatase, receptor type, R	protein tyrosine/serine/threonine phosphatase activity

4.2. ECM complex

The degree to which trophoblasts cells invade maternal tissue during placentation depends on how well they can degrade and remodel the extracellular matrix. In our current study, the function category of ECM complex enriched 13 DEGs between IMs and inter-IMs, with 10 genes upregulated and 3 genes downregulated in IMs compared to inter-IMs. Upregulated genes included ADAMTS4, MMP7, ADAMTS8, S100A12, COL15A1, ADAM8, MMP8, ADAMTS9, MMP13, CASK, and downregulated genes included PLCH, MIB2 and C1QTNF7. A disintegrin and metalloproteinases (ADAMs) with thrombospondin motifs (ADAMTSs), which are traditionally thought to control ECM proteolysis, were found to be highest in the pregnant uterus around the time of implantation and spatially highest at the site of implantation [38]. In human placentas, ADAMTS4 and 5 were most highly expressed in the first trimester when placental invasion is most pronounced [39]. ADAMTS9 increases in the mouse uterus during implantation and mainly localized to the vascular endothelial cells of the endometrium [40]. Although the ADAMTS subtype was found to be over-expressed during embryo implantation, the possible mechanisms and pathways during trophoblast cell invasion and function need further investigation.

Matrix metallopoteinases (MMPs) play critical roles in controlling invasion by trophoblast cells. MMP-7 and -8 were found in human early pregnancy decidual stromal cells and extravillous cytotrophoblasts, and MMP7 expression was decreased in women with recurrent implantation failure compared with those exhibiting normal fertility [41]. A study in rats showed that MMP7 was increased in IMs compared to inter-IMs [42]. However, MMP8 and MMP13 have not been detected in the pre- or peri-implantation mouse uterus [43,44]. MMP13 was only found in human extravillous cytotrophoblasts. Little information was gathered about the function and mechanism of MMP subtypes on embryo invasion and implantation, so more work needs to be done to verify if they are necessary during embryo implantation.

4.3. Cytokine-cytokine receptor interaction

In our study, the significantly differentially expressed cytokines between IMs and inter-IMs included the CXC subfamily (IL8RB, IL8, CXCL14), CC subfamily (CCL13, CCR1, CCL8), hematopoietins (IL6, IL11, OSMR, LIF, CSF3, CSF3R), IL-3RB (IL3RA), PDGF family (HGF), TNF family (TNFRSF21, TNFRSF12A), TGF-β family (IL1B), and IL-17 family (IL17RA). CXCL14 was reported to be involved in chemo-attraction, angiogenesis and cancer [45]. Targeted deletion of the CXCL14 gene in the pregnant mouse uterus impaired the population of uNK cells [46], which are considered to regulate trophoblast invasion by production of IL8 and interferon-inducible protein-10 chemokines [47]. Kuang [48] also revealed that CXCL14 was significantly upregulated at IMs compared to inter-IMs on pregnancy day 5 in mice. The levels of IL8, IL6 and IL17 were shown to be higher in normal fertile human endometrial cells than in uteri from patients with repeated implantation failure uteri [49]. CSF3 (colony-stimulating factor3), which is secreted from both immune and placental trophoblast cells, acts through specific membrane receptors, via JAK-STAT signaling pathways in an endocrine, paracrine or autocrine manner [50]. Studies have shown that CSF3 is important in promoting proliferation of trophoblast cells [51]. Administration of the cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF), prevents immune-mediated abortions in mice and improves pregnancy rates in women with a history of recurrent abortions [51,52], however, the specific pathway and mechanism of this effect is not clear.

4.4. Hormone related genes

We also investigated the involvement of hormone-regulated genes and pathways in DEGs between IMs and inter-IMs. Enriched pathways

Table 3
Top ten GO terms and enriched genes in IMs vs. Inter-IMs at P < 0.01.

Category	Terms	P value	Genes
Biological process	Immune response	2.30E-10	CCL15↑, IL6↑, SEPT9↑, CCL13↑, WDR78↓, HOPX↑, CXCL14↑, CSF3↑, CXCL1↑, NFKBID↓, CCL8↑,LIF↑, CD1↑, IL3RA↓, IL1B↑, APLN↑, CXCL8↑, TTC3↓
	Locomotion	4.06E-05	CCL8↑, CXCL1↑, NAV2↓, EMILIN2↑, CCL13↑, WDR78↓, CEBPD↑, CCL15↑, GAS8↓, MIIP↓, P2RY6↑, CCDC57↓, TPM4↑, IL8↑, LAMA3↑, IL1B↑, MAP2K1↑
	Response to external stimulus	0.000226	SHE↓, IL3RA↓, IL1B↑, IL8↑, TPM4↑, CCDC57↓, ARL15↓, BORA↑, CXCL1↑, CASK↑, CCL15↑, PITPNM2↑, WDR78↓, CEBPD↑, CCL13↑, CXCL14↑, NAV2↓, SLC41A2↓, CCL8↑
	Cell adhesion	0.00092	IL1B↑, CASK↑, EMILIN2↑, IL8 ↑, LAMA3↑
Molecular function	Cytokine activity	1.83E-15	CXCL1↑, CSF3↑, LIF↑, IL8↑, IL1B↑, CCL8↑, CCL13↑, WDR78↓, CCL15↑, IL6↑
	Chemokine activity	6.91E-11	CCL15↑, WDR78↓, CCL13↑, IL8↑, CXCL14↑, CCL8↑,
	Receptor binding	3.15E-08	TPD52L1↑, CXCL14↑, CSF3↑, TBX3↑, IL6↑, CASK↑, CCL15↑, CCL13↑, PTHLH↑, EMILIN2↑, WNT7↑, FAAP100↓, TLN↑, NFKBID↑, MAP2K1↑, IL1B↑, APLN↑, LIF↑, LAMA3↑, TESPA1↑, PRSS22↓, TPM4↑, NFKBID↑, CCL8↑, STC2↑, P2RX3↑, WDR78↓, CCDC57↓, FGG↑, NPPB↑, IL8↑
Cellular component	Interleukin-6 receptor complex	7.54E-05	IL6↑, CSF3↑
	Extracellular matrix	7.92E-05	ADAMTS4↑, MMP7↑, ADAMTS8↑, S100A12↑, PLCH↓, COL15A1↑, ADAM8↑, MIB2↓, MMP8↑, C1QTNF7↓, ADAMTS9↑, MMP13↑, CASK↑
	Cytoskeleton	0.029072	CAGE1↓, LAMA3↑, TPM4↑, MAP7D2↓, GULP1↓, APOL6↓, CCDC57↓, ANGPT2↑, CASK↑, TNIP3↑, MAPT↓, ENTPD5↓, EMILIN2↑, TLN↑, EIF4G1↑, TPD52L1↑, RALGAPA2↓, NAV2↓, FCER2↑, TUBA↑, MAP7D3↑, MYO5C↓

included the prolactin signaling pathway, GnRH signaling pathway, ovarian steroidogenesis, and insulin signaling pathway. The effects of prolactin are mediated by a membrane bound receptor (PRLR), through which prolactin stimulates Jak/STAT and ERK/MAPK pathways to influence glandular epithelia function/secretions. Prolactin has been reported to induce termination of obligate diapause in mink [53]. Prolactin receptor is expressed in the endometrium, and its abundance is influenced by ovarian steroids [54]. PRLR mRNA and protein are low during diapauses and early reactivation of embryos but increased around the time of implantation [9], Prolactin could upregulate its own receptors in the mink uterus [54]. Several studies have shown that in mink uterine epithelial cells treated with prolactin, uterine polyamine levels were altered, and their in vitro coculture increased embryo survival and attachment [9]. Five DEGs were significantly enriched in prolactin signaling pathway, including ESR2, TH, SOCS5, FOS and MEK1. The estrogen of ovarian origin that supports implantation in the mouse uterus acts through the estrogen receptor-β(ESR2), and null mutations for genes encoding Esr2 have confirmed the critical contribution of steroid signaling for endometrial receptivity and implantation [55]. In rat model, the ability of the corpus luteum to respond to estrogen requires prolactin [56]. Recently Gubbay et al., demonstrated, Prolactin also induces the ERK/MAPK pathway within the secretory phase of the human endometrium [57], In the ERK/MAPK cascade, MEK1 and MEK2 are dual-specificity kinases responsible for the activation of the ERK1 and ERK2 kinases. Inactivation of Mek1 causes placental malformations resulting from defective proliferation and differentiation of the trophoblast cells and leading to a severe delay in the development and the vascularization of the mouse placenta, which explains the embryonic death [58]. However, these mechanisms have not yet been reported in mink.

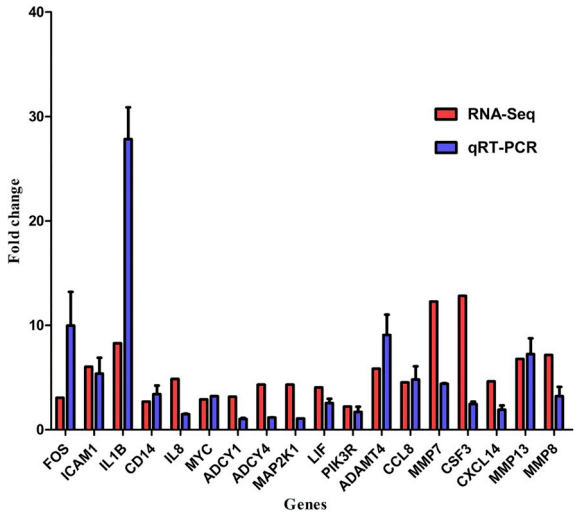

Fig. 1. Real-time Q-PCR validation of RNA-Seq data. Data is shown as (mean ± SD) fold-changes between IMs and inter-IMs. Statistical significance was reached at P < 0.05 for all tests. Blue bars represent qRT-PCR, while red bars represent RNA-Seq.

4.5. Gene associated with vascular development

Previous studies have shown that vascular development in attachment sites is essential for successful embryonic implantation, formation of the placenta and fetal development [59]. In the functional analysis, we observed that genes detected at a greater abundance in IMs than

Table 4
Most enriched pathways in IMs vs. Inter- IMs.

KEGG pathway	P value	Count	Genes
Malaria	5.76E-07	10	CSF3↑, IL6↑, IL8↑, SELE↑, ITGAL↑, ICAM1↑, HGF↑, IL1B↑, SELP↑, THBS1↑
Cytokine-cytokine receptor interaction	3.06E-06	18	CSF3↑, IL6↑, IL3RA↓, IL8↑, LIF↑, CSF3R↑, CCL13↑, CCL8↑, IL11↑, TNFRSF21↑, IL17R↑, HGF↑, IL1B↑, OSMR↑, CXCL14↑, TNFRSF12A↑, IL8RB↑, CCR1↑
Pyrimidine metabolism	2.41E-05	13	RPC7↑,ENTPD3↑, UPP1↑, NME1↑, CTPS1↑, trxB↑, ENTPD5↓, CD73↑
Jak-STAT signaling pathway	8.28E-05	13	CSF3↑, IL6↑, PIK3R5↑, LIF↑, CSF3R↑, IL27RA↑, IL11↑, SOCS5↑, OSMR↑, CDKN1A↑, MYC↑, LIF↑, PIM1↑
TNF signaling pathway	0.000322	8	IL6↑, PIK3R5↑, LIF↑, SELE↑, ICAM1↑, FOS↑, MAP2K1↑, BCL3↑
Chemokine signaling pathway	0.000695	10	ADCY4↑, PIK3R5↑, ADCY1↑, FGR↑, CCL8↑, GNGT2↑, CXCL14↑, MAP2K1↑, IL8↑, CCR1↑
Hematopoietic cell lineage	0.000123	13	CSF3↑, IL6↑, CD1↑, FCER2↑, CSF3R↑, IL11↑, IL1B↑, ITGA5↑, IL3RA↓, ITGAM↑, CD1D↑
Pertussis	0.002974	8	IL6↑, IL8↑, FOS↑, IL1B↑, ITGA5↑, C5↑,ITGAM↑, CASP1↑
Drug metabolism - other enzymes	0.00228	1	Udp↑,
Mineral absorption	0.011527	3	SLC39A4↓, SLC11A↑

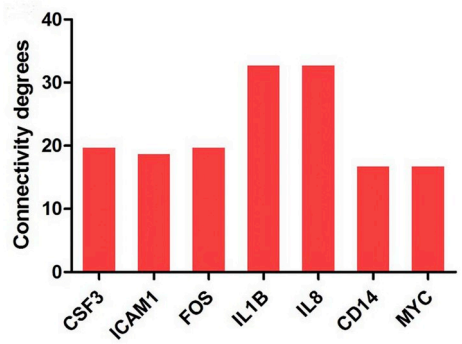

Fig. 2. Connectivity degrees of hub genes in the PPI network.

inter-IMs related to vascular development, e.g., mitogen-activated protein kinase kinase 1 (MAP2K1), interleukin 1 (IL1B), apelin (APLN), angiopoietin 2 (ANGPT2) and metallopeptidase inhibitor family (TIMP1). MAP2K1 encodes dual specificity kinases responsible for ERK/MAP kinase activation, and in mice, loss of MAP2K1 function causes embryonic lethality due to placental defects. It has been reported that in sheep the VEGF- and FGF2-stimulated fetoplacental artery endothelial cell proliferation is mediated via the MAP2K1 and phosphoinositide 2-kinase (PI3K)/AKT1 signaling pathways [60].

4.6. Regulatory network analysis

In the present study, we identified 7 hub genes, which are likely more important than other genes due to their key positions in the network. These genes are CSF3, ICAM1, FOS, IL1B, IL8, CD14 and MYC. Among these genes, CSF3, IL8 and IL1B are involved in immune response according to the GO function classification. Proto-oncogene FOS is a transcription factor that encodes nuclear proteins known to be involved in cell proliferation and differentiation, and it may have a critical function in cellular interactions that regulate embryonic development [61]. In studies on mice, c-myc protein levels at IMs was much higher than at inter-IMs [62]. c-myc expression was lower in pre-eclampsia than those in normal pregnancy further supports its important role during embryo implantation [63]. c-myc is up-regulated by prolactin through the PI3K/AKT pathway [64]. In the KEGG database, myc is downstream of the PI3K/AKT pathway and regulates cell cycle progression. Further study needs to be done to identify the role and pathway of myc in embryo invasion and implantation. ICAM1 is intercellular adhesion molecule, expressed on endothelial cells, where they bind to β2-integrin ligands on leukocytes [65]. In a study on rats, ICAM1 increased at the time of implantation, suggesting a cell adhesion role in embryo-uterine interaction [66]. CD14, a glycoprotein receptor, has a relevant role in lipopolysaccharide (LPS)-mediated cellular activation [67]. It has been reported that LPS amplified the inflammatory response by inducing CD14 expression in endometrial glands [68].

5. Perspectives

The results of IMs and inter-IMs transcriptome profiling described here indicate that multiple regulatory pathways are activated during embryo invasion into the endometrium. And as a result failure of implantation, several of the genes and pathways identified as being involved in pathological states, including staphylococcus aureus infection, prion diseases, legionellosis, African trypanosomiasis, salmonella infection, amebiasis and cancer, may contribute to endothelial dysfunction. In addition to genes involved in the regulation of the maternal immune system, cell adhesion, migration and invasion, nutrient transport, cell proliferation, apoptosis, proteolysis and angiogenesis, these molecular dysregulations in the endometrium may result in abnormal placentation. Hormones stimulate the formation of a suitable physiologic milieu for implantation of the embryo and early placentation. Acquired diseases or perturbations of the maternal physiology during embryo implantation, especially during the crucial early placentation period, can affect endometrial function and fetal development. More research efforts are needed to amplify and validate the current study with details of the localization and function of important genes and proteins, which may provide a more precise understanding of trophectoderm proliferation, differentiation and invasion, as well as fetus-maternal interactions and promotion of successful pregnancy in mink.

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Conflicts of interest

The authors report no conflict of interest.

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Appendix A. Supplementary data

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