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Research Article
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Revealing the expression profile of genes that encode the Subcortical Maternal Complex in human reproductive failures

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Abstract

The Subcortical Maternal Complex (SCMC) is composed of maternally encoded proteins required for the early stages of embryo development. Here we aimed to investigate the expression profile of the genes that encode the individual members of the SCMC in human reproductive failures. To accomplish that, we selected three datasets in the Gene Expression Omnibus repository for differential gene expression (DGE) analysis, comprising human endometrial and placental tissues of patients with recurrent implantation failure (RIF) or recurrent pregnancy loss (RPL). The SCMC genes *KHDC3L*, *NLRP2*, *NLRP4*, *NLRP5*, *OOEP*, *PADI6*, *TLE6*, and *ZBED3* were included in the DGE analysis, as well as *CFL1* and *CFL2* that connect the SCMC with the actin cytoskeleton. Additionally, differential co-expression analysis and systems biology analysis of gene-gene co-expression were performed for *KHDC3L*, *NLRP5*, *OOEP*, and *TLE6*, demonstrating gene pairs differentially correlated under the two conditions, and the co-expression with genes involved in immune response, cell cycle, DNA damage repair, embryo development, and male reproduction. Compared to control groups, *NLRP5* demonstrated upregulation in the endometrium of RIF patients, and *KHDC3L* was upregulated in the fetal placental tissue of RPL patients, shedding light on the importance of considering SCMC genes in reproductive failures.

Keywords: Decidua, chorionic villus, maternal effect genes, transcriptome, Pearson's correlation.

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Introduction

Infertility is the failure to establish a clinical pregnancy after 12 months of regular and unprotected sexual intercourse, affecting 8-12% of reproductive-aged couples worldwide (Vander Borght and Wyns, 2018). Many factors may lead to infertility, being manifested in different ways, according to the impact on the processes related to human reproduction, whether of maternal, paternal, and/or embryonic origin (Carson and Kallen, 2021). Fertilization failure, embryo arrest, and embryonic implantation failure are some of the reasons for the inability to initiate gestation. However, even once the pregnancy is achieved, its maintenance depends on the correct communication between maternal and embryonic tissues, and abnormalities during this period can lead to pregnancy losses (Ashary *et al.*, 2018).

Recurrent implantation failure (RIF) is the lack of implantation after the transfer of several embryos through assisted reproductive technologies (Franasiak *et al.*, 2021), whilst recurrent pregnancy loss (RPL) is the failure of two or more clinically recognized pregnancies before 20-24 weeks of gestation (Dimitriadis *et al.*, 2020). However, there is no consensus on the definition of RIF and RPL, varying according to the published guidelines. Both conditions may be related with disturbances in the maternal immune system, genetics of the embryo, anatomic factors, hematologic factors, reproductive tract microbiome, and endocrine environment, as well as endometrial-embryo asynchrony (Dimitriadis *et al.*, 2020; Franasiak *et al.*, 2021).

The processes involved in early embryo development are regulated and coordinated simultaneously to ensure the generation of a competent embryo capable of sustaining the

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implantation process and the maintenance of pregnancy (Conti and Franciosi, 2018). During this critical period, specific patterns of gene expression are paramount for regulating cellular proliferation and differentiation, being pivotal for the correct embryo development (Shahbazi and Zernicka-Goetz, 2018). In addition, proper gene expression in the maternal tissues during the time of implantation and pregnancy is also necessary for the changes that this period requires in the maternal reproductive environment (Ashary *et al.*, 2018).

Before the embryo genome activation, the initial development relies almost entirely upon maternal-effect-genes, which have important roles during embryogenesis, such as in the elimination of maternal mRNAs and proteins, epigenetic remodeling in oocytes and early embryos, as well as embryo genome activation (Conti and Franciosi, 2018). Recently, a Subcortical Maternal Complex (SCMC), comprising proteins encoded by maternal effect genes, was identified in mice (Li *et al.*, 2008a) and humans (Zhu *et al.*, 2014), demonstrating fundamental roles in early embryogenesis. Four proteins compose the SCMC: KHDC3L, NLRP5, OOEP, and TLE6. However, the sum of the four proteins (~255 kDa) is smaller than the estimated molecular weight of the SCMC (~669-2000 kDa), hypothesizing that other proteins may be part of the complex, such as the candidates NLRP2, NLRP4F, PADI6, and ZBED3 (Bebbere *et al.*, 2021). In addition, it was demonstrated that the SCMC interacts with the actin cytoskeleton through Cofilin (CFL), regulating symmetric cell divisions of mouse zygotes (Yu *et al.*, 2014).

The SCMC appears to work as a maternal functional module regulating mammalian early embryogenesis (Lu *et al.*, 2017), however, we hypothesized the individual members of the SCMC could have other roles in human reproduction in addition to embryonic development. Although the SCMC is confirmedly present only in oocytes and early embryos (Li *et al.*, 2008b; Zhu *et al.*, 2014), it is not settled whether the proteins of the SCMC could act as single molecules in other tissues, not being aggregated to form the multi-protein complex. Literature reports based on the evaluation of conditions such as male fertility (Rockenbach *et al.*, 2023), and imprinting disorders (Eggermann *et al.*, 2021), have helped to instigate this hypothesis. However, not limited to tissue variability, we speculate whether the individual members of the SCMC could have roles in processes such as embryonic implantation and even in later steps, such as the maintenance of pregnancy.

It is well-defined that pregnancy initiation and continuation are regulated by different molecular mechanisms that must be correctly orchestrated between maternal and embryofetal tissues (Ashary *et al.*, 2018). Since the SCMC expression is pivotal for the embryonic genome activation and other initial steps of the pregnancy initiation (Lu *et al.*, 2017), it is coherent to hypothesize that its inactivation might result in an implantation failure (IF). Nevertheless, literature is scarce in regard to the effects of the SCMC in the later gestational period. If the SCMC proteins, acting as a complex or as single molecules, have a role in placentation and endometrial receptivity, it is also feasible to suggest they might be implicated in the recurrent pregnancy loss (RPL) etiology. Therefore, we analyzed the gene expression profile of the SCMC genes, as well as *CFL1* and *CFL2* in endometrial and placental tissues of patients with RIF or RPL through publicly available transcriptomes.

Material and Methods

Gene expression analysis

The expression profile of the SCMC genes in RIF or RPL patients was evaluated through differential gene expression (DGE) and differential co-expression analyses of transcriptome data available in the Gene Expression Omnibus (GEO) repository (Edgar *et al.*, 2002; Barrett *et al.*, 2013). For each pathological condition (RIF or RPL), the comparisons were performed against a control group, considering for DGE analysis the gene expression of *KHDC3L, NLRP5, OOEP, TLE6, CFL1, CFL2, NLRP2, NLRP4, PADI6,* and *ZBED3*, and for differential co-expression analysis the expression of *KHDC3L, NLRP5, OOEP,* and *TLE6.*

Obtention of transcriptome data

For datasets search in the GEO, the keywords "implantation failure", "pregnancy loss", "endometrium", "placenta", "chorionic villus", and "decidua" were used, filtering by Entry type (Series), Organism (*Homo sapiens*), and study type (Expression Profile by Array or Expression Profile by Throughput Sequencing). Only studies performed in consolidated platforms, containing the raw data, experimental design, and well-described sample groups were included.

Following these criteria, three studies were selected, covering endometrium samples of patients with RPL or RIF, as well as placental tissue (chorionic villus or decidua) of RPL patients: GSE26787 (Lédée *et al.*, 2011), GSE121950 (Huang *et al.,* 2018), and GSE113790 (Yu *et al.*, 2018). In the studies selected, the RPL definition was: having at least three pregnancy losses between 6 and 12 weeks of gestations (GSE26787) or two or more consecutive pregnancy losses before 20 weeks of gestations (GSE121950 and GSE113790). RIF was defined as the absence of pregnancy despite the transfer of at least ten embryos over several assisted reproductive cycles (GSE26787). The Control group for endometrial sample of patients with RIF or RPL was fertile patients (successfully delivered after the first or second attempt of IUI or IVF/ICSI related to a male infertility diagnosis). The Control group for chorionic villus or decidua sample of RPL patients consisted of women who underwent legal termination of an apparently normal early pregnancy, without medical reasons, history of pregnancy loss or any pregnancy complication. Additional information about the datasets is available in the supplementary material (Table S1).

Differential gene expression analysis

The DGE analysis was conducted in the R environment (v.3.6.3). For the studies comprising RNA-Seq data, sequence alignment was performed through the Galaxy Europe server (Jalili *et al.*, 2020), using the HISAT2 (Kim *et al.*, 2019) alignment tool against the human reference genome hg38 and transcript count was performed through featureCount tool (Liao *et al.*, 2014). The parameters for RNA-Seq data alignment and transcript count were the default ones, and the

alignment rate was above 80% for all the samples analyzed. The DGE was calculated in the aligned transcriptomes using the *edgeR* (v.3.28.1) (Robinson *et al.*, 2010) package. Considering microarray data, the packages *affy* (v.1.64.0) (Gautier *et al.*, 2004) and *limma* (v.3.42.2) (Ritchie *et al.*, 2015) were used to evaluate the DGE. RNA-Seq data was normalized through the trimmed mean of M values (TMM) and microarray data by robust multi-array average (RMA). The DGE results are demonstrated as values of $\log_2$ fold-change (logFC) and adjusted P-value for false discovery rate (FDR), being the DGE considered statistically significant when identified a gene with both $\log_2$ fold-change (logFC) $\geq |1.0|$ and adjusted P-value ≤ 0.05. The heatmaps were generated in the R environment through the *ggplot* package (v.3.3.5).

Differential co-expression analysis

Additional to the DGE analysis, a differential co-expression analysis was performed considering the basal gene expression of *KHDC3L*, *NLRP5*, *OOEP*, and *TLE6* in control and RIF or RPL patients. Gene-gene co-expression was evaluated using Pearson's correlation coefficient (Pearson's r) through the *diffcoexp* package (v.3.17) in the R environment. According to Pearson's r, a negative correlation coefficient means one gene is upregulated and the other is downregulated; hence, there is an inverse expression between gene pairs. In contrast, positive correlation coefficients mean both genes are upregulated or downregulated. Gene-pairs co-expression was considered moderately correlated when Pearson's r was $\geq |0.5|$ and highly correlated when Pearson's r was $\geq |0.8|$. In this study, Pearson's r was calculated for control samples and then for fertility issues samples (RIF or RPL). The differential correlation between control vs. affected group was calculated through Fisher's Z transformation method and P-Values < 0.05 was set as significant. Due to the small number of gene-pairs evaluated, no adjustment in the P-Values were performed, but q-Values are presented in Table 1. Hence, gene-pairs were considered differentially co-expressed when there was a significantly different correlation coefficient under the two conditions. As in DGE, the heatmaps for the differential co-expression analyses were generated in the R environment through the *ggplot* package.

Systems biology analysis

To better elucidate the roles of the SCMC genes in multifactorial conditions such as RIF and RPL, a systems biology approach was conducted for the four validated components of the SCMC: *KHDC3L*, *NLRP5*, *OOEP*, and *TLE6*. A co-expression network was assembled in the Cytoscape (v. 3.8) using the GeneMania application (Montojo *et al.*, 2010), considering only the co-expressed genes filter.

Table 1 – Statistical analysis for the differential co-expression results. Cor (Pearson's correlation coefficient); diff (differences between control and cases); RPL (recurrent pregnancy loss); RIF (recurrent implantation failure); P-Values ≤ 0.05 were statistically significant.

RECURRENT IMPLANTATION FAILURE VS. CONTROL (ENDOMETRIUM)									
Gene pairs	cor.Control	cor.RIF	cor.diff	p.Control	p.RIF	p.diffcor	q.Control	q.RIF	q.diffcor
NLRP5 and KHDC3L	-0.723796175	0.298373579	1.022169754	0.166844543	0.625813633	0.221214199	0.333689086	0.914828318	0.485936692
OOEP and KHDC3L	0.524053513	-0.066943717	-0.59099723	0.364696684	0.914828318	0.51636701	0.436785483	0.914828318	0.619640412
TLE6 and KHDC3L	0.560272726	0.233327983	-0.326944744	0.325951115	0.705635663	0.692455908	0.436785483	0.914828318	0.692455908
OOEP and NLRP5	-0.835869437	0.748757795	1.584627232	0.07782624	0.145340872	0.029448694	0.333689086	0.872045231	0.176692164
TLE6 and NLRP5	-0.459018994	0.413211003	0.872229998	0.436785483	0.489264214	0.349507554	0.436785483	0.914828318	0.524261331
TLE6 and OOEP	0.753369236	-0.184752233	-0.93812147	0.14146437	0.766111309	0.242968346	0.333689086	0.914828318	0.485936692
RECURRENT PREGNANCY LOSS VS. CONTROL (ENDOMETRIUM)									
Gene pairs	cor.Control	cor.RPL	cor.diff	p.Control	p.RPL	p.diffcor	q.Control	q.RPL	q.diffcor
NLRP5 and KHDC3L	-0.768283904	0.152667252	0.920951156	0.129141389	0.806375766	0.242002513	0.468437608	0.909887142	0.889762906
OOEP and KHDC3L	0.585454653	-0.070833752	-0.656288404	0.299672502	0.909887142	0.45828449	0.599345004	0.909887142	0.889762906
TLE6 and KHDC3L	0.277811088	0.400221583	0.122410495	0.65088408	0.504373014	0.889762906	0.781060896	0.909887142	0.889762906
OOEP and NLRP5	-0.73609005	-0.184501747	0.551588303	0.156145869	0.766424755	0.450097613	0.468437608	0.909887142	0.889762906
TLE6 and NLRP5	0.098918468	-0.336636046	-0.435554514	0.874258793	0.579620628	0.653044484	0.874258793	0.909887142	0.889762906
TLE6 and OOEP	0.385077984	0.533510827	0.148432843	0.522105678	0.354476506	0.85006775	0.781060896	0.909887142	0.889762906

Table 1 – Cont.

RECURRENT PREGNANCY LOSS VS. CONTROL (CHORIONIC VILLUS)									
Gene pairs	cor.Control	cor.RPL	cor.diff	p.Control	p.RPL	p.diffcor	q.Control	q.RPL	q.diffcor
NLRP5 and KHDC3L	0.977945255	-0.913894241	-1.891839495	0.000724254	0.0108021	3.28E-06	0.002172762	0.017799721	9.83E-06
OOEP and KHDC3L	0.199428479	0.90968646	0.710257981	0.704823088	0.01186648	0.105011012	0.704823088	0.017799721	0.105011012
TLE6 and KHDC3L	-0.942535195	0.972483894	1.915019089	0.004858426	0.001125287	1.82E-06	0.009716851	0.003375862	9.83E-06
OOEP and NLRP5	0.389749943	-0.997796689	-1.387546632	0.444977572	7.28E-06	0.000180062	0.533973086	4.37E-05	0.000360123
TLE6 and NLRP5	-0.988582995	-0.848216272	0.140366723	0.000194778	0.032809031	0.103260108	0.001168667	0.039370837	0.105011012
TLE6 and OOEP	-0.514000802	0.829849741	1.343850543	0.296897486	0.040963646	0.031522489	0.44534623	0.040963646	0.047283734
RECURRENT PREGNANCY LOSS VS. CONTROL (DECIDUA)									
Gene pairs	cor.Control	cor.RPL	cor.diff	p.Control	p.RPL	p.diffcor	q.Control	q.RPL	q.diffcor
NLRP5 and KHDC3L	0.060448613	-0.951645102	-1.012093715	0.961493743	0.198783852	1	0.961493743	0.669530442	1
OOEP and KHDC3L	0.638595927	-0.836823504	-1.475419431	0.559031102	0.368820066	1	0.746032173	0.669530442	1
TLE6 and KHDC3L	-0.860901692	0.764113321	1.625015013	0.339800266	0.446353628	1	0.746032173	0.669530442	1
OOEP and NLRP5	0.806737213	0.628175571	-0.178561642	0.402462641	0.567603918	1	0.746032173	0.681124702	1
TLE6 and NLRP5	-0.559881268	-0.925333648	-0.36545238	0.621693477	0.247569776	1	0.746032173	0.669530442	1
TLE6 and OOEP	-0.941289345	-0.286263143	0.655026202	0.219230836	0.815173694	1	0.746032173	0.815173694	1

Results

An upregulation of *NLRP5* was observed in the endometrium of patients with RIF compared to the control group (logFC = 3.025; adjusted P-Value = 0.014). Although it was not statistically significant in the other three analyses, *NLRP5* was upregulated in the four scenarios evaluated. Considering the placental tissue, an upregulation of *KHDC3L* was demonstrated in the chorionic villus of RPL patients when compared to the control group (logFC = 3.008; adjusted P-Value = 0.003) (Figure 1).

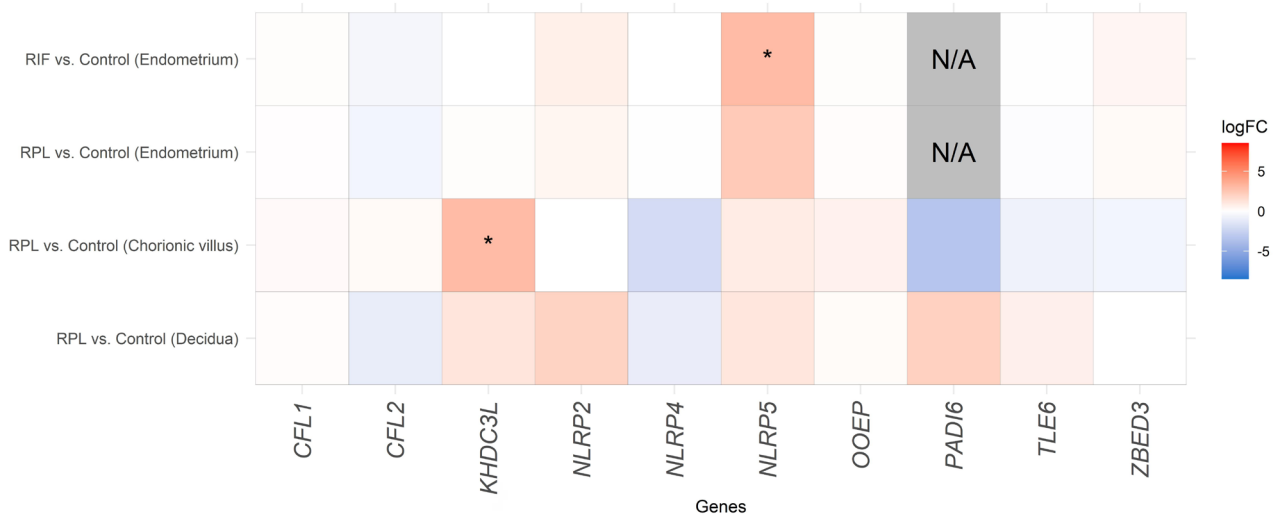

Figure 1 – Heatmap of the Subcortical Maternal Complex differential gene expression in endometrial and placental tissues in human reproductive failures. RIF (recurrent implantation failure); RPL (recurrent pregnancy loss); N/A (not available); LogFC (Log_2 Fold Change); * means statistically significant differences between groups ($p \leq 0.05$).

Interestingly, no statistically significantly altered genes were observed in the placental samples of maternal origin (decidua) (Adjusted P-Value > 0.05); however, some logFC were increased, demonstrating a differential expression might be present, but without statistical power to confirm it. The values of logFC and adjusted P-Values for all the SCMC genes analyzed are available in Table 2.

Pearson's r was calculated to evaluate the co-expression between gene-pairs, considering the four genes of the SCMC complex. It was observed that the gene expression correlation between *KHDC3L*, *NLRP5*, *OOEP*, and *TLE6* is lost in the endometrium of RIF patients compared to the control group (Figure 2A), although it was not statistically different when applying Fisher's-Z transformation. Except for *TLE6*

Table 2 – Statistical analysis for the differential gene expression results. LogFC (log_2 fold-change); FDR (false-discovery rate); NA (not available); the DGE was considered statistically significant when both logFC ≥ |1.0| and adjusted P-Value ≤ 0.05.

Study	Comparison	Genes	logFC	Adjusted P-value (FDR)
GSE26787	Recurrent implantation failure vs. Control (Endometrium)	*CFL1*	0.1026367	0.5229698
		CFL2	-0.498079897867953	0.251416316493106
		KHDC3L	-0.001182168	0.9962905
		NLRP2	0.681588601771906	0.681588755422925
		NLRP4	0.009650816	0.9714375
		NLRP5	**3.02522473023772**	**0.0141471070182118**
		OOEP	0.111747	0.8106413
		PADI6	NA	NA
		TLE6	0.02474961	0.9497394
		ZBED3	0.379691270618091	0.559698817131491
	Recurrent pregnancy loss vs. Control (Endometrium)	*CFL1*	0.0786863159624627	0.59939475537244
		CFL2	-0.582603971710068	0.277270727132137
		KHDC3L	0.0811707017617804	0.75759904538884
		NLRP2	0.457898306429951	0.696673633291216
		NLRP4	0.0259633183126278	0.918007451161419
		NLRP5	2.34493710842825	0.251792093808216
		OOEP	0.117712311568029	0.787288812071011
		PADI6	NA	NA
		TLE6	-0.148942627713211	0.485773545319927
		ZBED3	0.259967817024501	0.549032896179686
GSE121950	Recurrent pregnancy loss vs. Control (Chorionic villus)	*CFL1*	0.207979556312718	0.39350378872339
		CFL2	0.211804830319393	0.704923759930941
		KHDC3L	**3.00853375453727**	**0.00293672701411503**
		NLRP2	-0.00893939223730675	1
		NLRP4	-2.02451760996167	0.370388208966524
		NLRP5	0.858074625612382	0.575326063792963
		OOEP	0.646653028452488	0.542039977168646
		PADI6	-3.35312117601586	0.160290137066962
		TLE6	-0.71115056497841	0.249818659395077
		ZBED3	-0.541428483402332	0.605711244872257
GSE113790	Recurrent pregnancy loss vs. Control (Decidua)	*CFL1*	0.122166824419392	1
		CFL2	-1.03283267355495	0.442221956087787
		KHDC3L	1.09732315067292	0.954460730152779
		NLRP2	1.97492180673471	0.831821230943147
		NLRP4	-0.999040419492552	0.986849232233079
		NLRP5	1.08159364196709	0.789621847412384
		OOEP	0.182337399040928	1
		PADI6	2.03571376974121	0.831795991373028
		TLE6	0.69944683541296	0.815358493093605
		ZBED3	0.00907890935220243	1

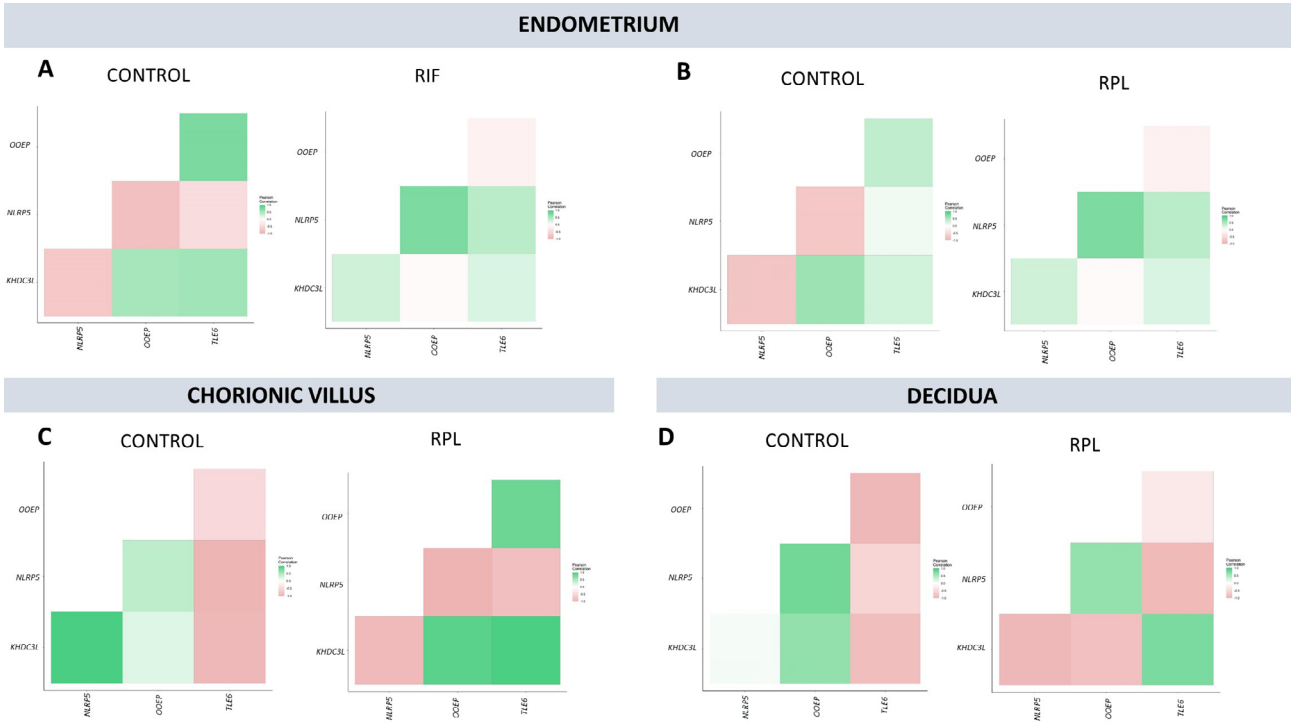

Figure 2 – Differential co-expression analysis for the four validated Subcortical Maternal Complex genes in the endometrium of control and RIF patients (A), endometrium of control and RPL patients (B), chorionic villus of control and RPL patients (C), and decidua of control and RPL patients (D). Positive correlations represented in green; negative correlations in pink; absence of correlation in white. RIF (recurrent implantation failure); RPL (recurrent pregnancy loss).

and *NLRP5* (Pearson's r < 0.5), all the other gene-pairs demonstrated a moderate or high correlation in the control group. However, in RIF patients these correlations were lost, except for *OOEP* and *NLRP5,* which significantly inverted the correlation pattern, from a high inverse correlation to a moderate positive correlation (Control = -0.84 vs. RIF = 0.75, P-Value = 0.03). Considering the endometrium of RPL patients, no statistically significant differences in SCMC genes' co-expression were observed (Figure 2B).

Comparing the chorionic villus of RPL and control patients, it was observed a statistically significant different correlation between *NLRP5* and *KHDC3L* (Control = 0.98 vs. RPL = -0.91, P-Value = 3,28E-06), *TLE6* and *KHDC3L* (Control = -0.94 vs. RPL = 0.97, P-Value = 1.82E-06), *OOEP* and *NLRP5* (Control = 0.39 vs. RPL = -0.99, P-Value = 0.0002), and *TLE6* and *OOEP* (Control = -0.51 vs. RPL = 0.83, Adjusted P-Value = 0.03) (Figure 2C). Interestingly, in the placental maternal tissue (decidua) it was not observed statistically significant differences in gene expression correlation between RPL and control patients (Figure 2D). The statistical analysis results for differential co-expression analysis are available in Table 1.

Gene-gene co-expression analysis for *KHDC3L*, *NLRP5*, *OOEP*, and *TLE6* was also evaluated through systems biology strategy. It was demonstrated the SCMC genes interact with genes related to DNA damage response and repair, embryo development, immune response, cell division, chromosome segregation, and male reproduction (Figure 3). The four SCMC genes were co-expressed with genes related to male reproduction, especially *OOEP* which was also co-expressed with genes located in the Y chromosome. Additionally, except for *OOEP*, all the other genes were co-expressed with genes associated with immune response, and only *KHDC3L* was co-expressed with a gene related to DNA damage repair.

Discussion

Embryo implantation and maintenance of pregnancy depend on a competent blastocyst, receptive endometrium, and successful cross-talk between the embryonic and maternal interfaces (Ashary *et al.*, 2018). Here we demonstrated, through publicly available data, altered SCMC gene expression and co-expression patterns in RIF and RPL patients. Compared to the control groups, an upregulation of *NLRP5* was demonstrated in the endometrium of patients with RIF, as well as an upregulation of *KHDC3L* in the chorionic villus of RPL patients. Additionally, we demonstrated that *KHDC3L*, *NLRP5*, *OOEP*, and *TLE6* are being co-expressed with genes involved in processes required for proper embryo development and gestational maintenance, such as immune response, cell proliferation, and DNA damage repair.

The SCMC exerts several functions during early embryo development, being required for embryo progression beyond the first cell divisions (Li *et al.*, 2008a; Zhu *et al.*, 2014). However, studies have demonstrated alterations in SCMC genes associated with later reproductive problems, such as recurrent hydatidiform mole (Ji *et al.*, 2019), RPL (Zhang *et al.*, 2019), and multilocus imprinting disorders (Docherty *et al.*, 2015). Although the molecular mechanisms behind reproductive disorders and SCMC genes remain poorly understood, it is feasible to suggest that SCMC gene expression is important not only during early embryogenesis but also later in pregnancy.

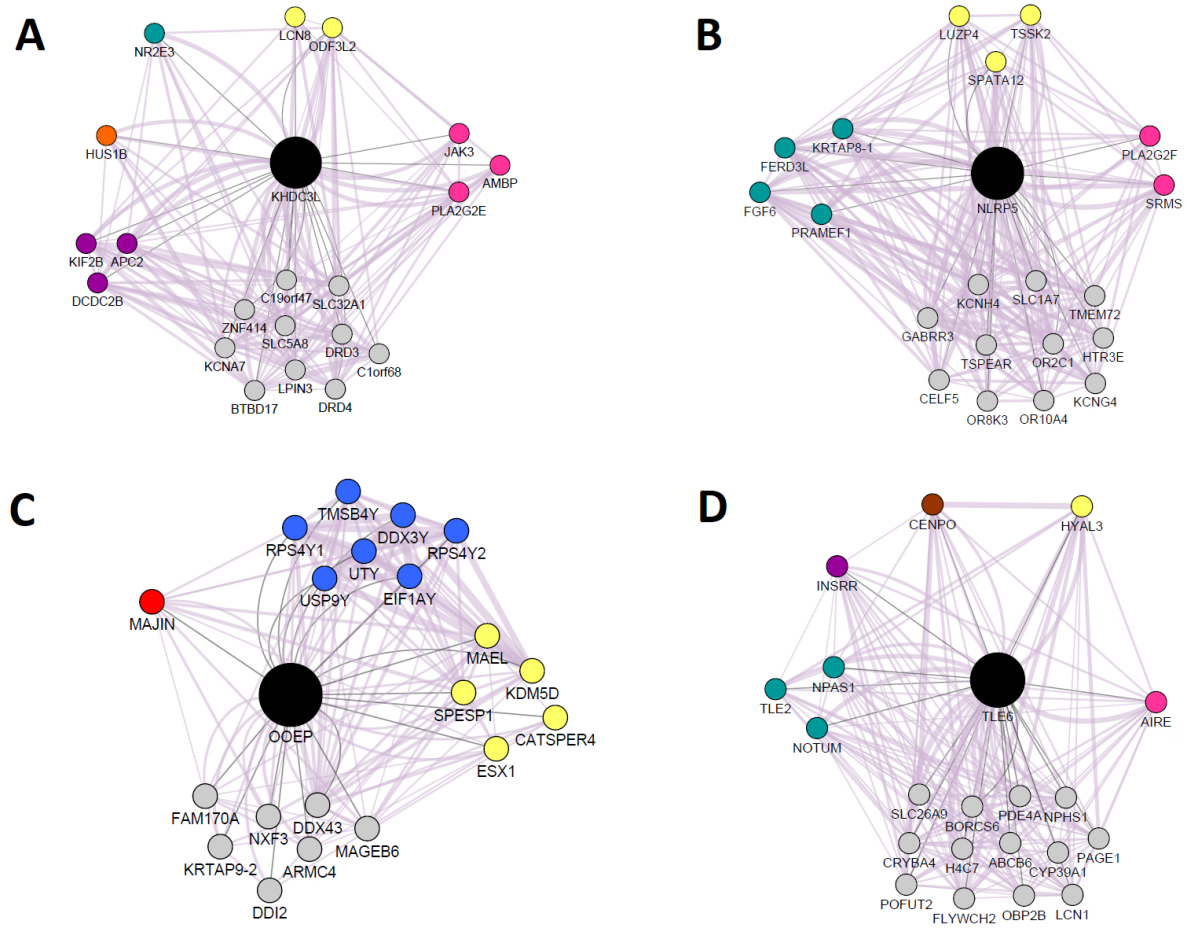

Figure 3 – Co-expression network for the four validated Subcortical Maternal Complex genes. A) *KHDC3L* co-expression network; B) *NLRP5* co-expression network; C) *OOEP* co-expression network; D) *TLE6* co-expression network. Nodes of different colors means distinct biological functions and edges connecting nodes represent genes that are co-expressed. Pink: genes related to immune response; yellow: male reproduction; green: embryonic development; purple: cytoskeleton organization; orange: DNA damage response; brown: cell cycle and chromosome segregation; red: meiosis; blue: located in Y chromosome; grey: other functions.

During the implantation process, the receptive endometrium is modified by embryonic signals accompanied by substantial morphological, molecular, and immunological changes required for proper embryo implantation and further maintenance of pregnancy (Ashary *et al.*, 2018). We demonstrated an upregulation of *NLRP5*, a member of the SCMC, in the endometrium of RIF patients in comparison to the control group, as well as *NLRP5* co-expression with genes related to immunological processes. Early pregnancy modulates the expression of the NLR family in ovine lymph nodes (Zhao *et al.*, 2022), evidencing a role for this protein family in maternal immune regulation during pregnancy. Considering NLR family of proteins have a role in the activation of pro-inflammatory cytokines (Platnich and Muruve, 2019) and embryo implantation is considered a pro-inflammatory reaction characterized by increased endometrial vascular permeability and trophoblast invasion (Kim and Kim, 2017), we postulate *NLRP5* upregulation in endometrial cells may affect embryo implantation through altered immunological regulation.

Gene variants in *NLRP5*, as well as in other genes of the SCMC, are associated with embryo arrest (Mu *et al.*, 2019; Xu *et al.*, 2020). Additionally to this phenotype, alterations in *NLRP5* have been associated with multilocus imprinting disorders in humans, a disturbance of multiple imprinting locus across the genome affecting metabolism, growth, and behavior (Docherty *et al.*, 2015; Sparago *et al.*, 2019). Epigenetic regulation of gene expression has a role in embryo implantation and gestational maintenance by regulating both embryo development and endometrial changes required for successful implantation (Munro *et al.*, 2010; Xu *et al.*, 2021). Although the mechanisms behind methylation defects associated with mutations in *NLRP5* remain to be elucidated, this gene could be involved in the epigenetic regulation of endometrial gene expression during embryo implantation. Therefore, we hypothesized that upregulation of *NLRP5* could be associated with epigenetic deregulation of genes important during the pro-inflammatory scenario necessary for the trophoblast invasion and embryo implantation, thereby affecting the activation of pro-inflammatory cytokines.

However, after embryo implantation and establishment of pregnancy, its maintenance depends not only on proper embryo development but also on the correct maternal-embryo communication (Ashary *et al.*, 2018). In this context, the correct formation of the placenta, an extraembryonic organ crucial for normal development and long-term health, is pivotal

for gestational maintenance (Knöfler *et al.*, 2019). Around 5-6 days after fertilization, the blastocyst develops and segregates into two cellular subtypes: the trophectoderm, which will differentiate to form the embryonic placental tissue – chorionic villus -, and the inner cell mass giving rise to the embryo proper (Knöfler *et al.*, 2019). After blastocyst implantation, placental development is initiated and trophectoderm-derived cells give rise to all trophoblast cell types of the future placenta (Woods *et al.*, 2018). In addition to the fetal-derived cells, the placental development is also dependent on the maternal uterine tissue into which the blastocyst is embedded after implantation (Woods *et al.*, 2018). The cells of the endometrium undergo decidualization, which is pivotal for supporting normal placentation and providing the proper environment for embryonic growth and survival (Woods *et al.*, 2018). Interestingly, we demonstrated an upregulation of *KHDC3L* in the chorionic villus of patients with RPL in comparison to the control group. Although *KHDC3L* mRNAs are rarely detected in human morulae, the transcript's level increases dramatically in the blastocyst, and like the other members of the SCMC, its location in the blastocyst stage is exclusive of the outer layer formed by the trophectoderm (Li *et al.*, 2008b; Zhu *et al.*, 2014). The specific localization of the SCMC during early embryo development could be associated with a role in lineage cell decisions during development and, in this context, *KHDC3L* could be related to the trophoblast cells proliferation and differentiation involved in placental development.

In addition, variants in *KHDC3L* have been associated with hydatidiform moles, an abnormal pregnancy characterized by abnormal trophectoderm proliferation and abnormal or no embryo development (Ji *et al.*, 2019; Demond *et al.*, 2019). This evidence demonstrates a possible role of *KHDC3L* in the trophectoderm proliferation and differentiation, which further could affect the placental development. Indeed, failures in placental formation can compromise embryonic growth and development, and abnormal placentation is a feature of diverse pregnancy complications such as pregnancy loss, stillbirth, intrauterine growth restriction, and preeclampsia (Knöfler *et al.*, 2019). *KHDC3L* variants have also been related to imprinting disturbance and genomic instability of early embryonic cells leading to reproductive failures, including RPL (Zhang *et al.*, 2019). Indeed, *KHDC3L* has a role in safeguarding genome integrity through homologous DNA repair (Zhang *et al.*, 2019) and stalled replication fork restart (Zhao *et al.*, 2018). Therefore, we hypothesized that upregulation of *KHDC3L* in chorionic villus could be associated with altered epigenetic regulation of genes related to DNA repair mechanisms, thereby disturbing trophoblast cell proliferation and differentiation, influencing the proper placental development.

Interestingly, considering *KHDC3L*, *NLRP5*, *OOEP*, and *TLE6*, it was observed that except for *TLE6* and *NLRP5*, all the other gene-pairs demonstrated a moderate or high correlation in the endometrium of control group. However, in RIF patients these correlations were lost. Furthermore, *OOEP* and *NLRP5* significantly inverted the correlation pattern, from a high negative correlation to a moderate positive correlation, which could be related to the upregulation of *NLRP5* demonstrated in the DGE results. Alterations in gene expression correlation were also observed between the gene-pairs *NLRP5* and *KHDC3L*, *TLE6* and *KHDC3L*, *OOEP* and *NLRP5*, and *TLE6* and *OOEP* in the chorionic villus of RPL patients. Considering the role of gene expression patterns during embryo development, the disrupted gene-gene co-expression demonstrated in RIF and RPL patients could influence the proper embryo implantation and gestational maintenance. Although we cannot confirm a causal association between RIF and RPL with altered co-expression patterns in the four validated SCMC genes, a transcriptional deregulation of these genes is present in these conditions and even if it is not associated with RIF and RPL, tertiary factors could be influencing this deregulation.

Additional to the differential co-expression analysis performed for *KHDC3L*, *NLRP5*, *OOEP*, and *TLE6*, we evaluated the co-expression of the SCMC members with other genes. Interestingly, the co-expression network demonstrated that the four validated SCMC genes are co-expressed with genes related to male reproduction. Interestingly, a previous work of our group evidenced *OOEP* downregulation in patients with teratozoospermia or non-obstructive azoospermia (Rockenbach *et al.*, 2023), evidencing possible new roles for the SCMC genes in both embryonic development and in female and male reproduction. Moreover, the results presented here shed light on a possible role of SCMC gene expression profile in later reproductive conditions, such as in post-implantation gestational events.

This study has some limitations, such as the lack of validation of the data analyzed and the absence of single-cell transcriptome studies. Functional analysis needs to be performed to demonstrate the mechanisms behind the gene expression alterations of *NLRP5* and *KHDC3L* in RIF and RPL patients, respectively, as well as in the altered co-expression patterns observed for these conditions. It is also important to highlight the biases of clinical differences between the datasets used in this study, such as the different definitions for RPL. However, the results presented here shed light on possible molecular mechanisms associated with reproductive failures and demonstrate the importance of considering the roles of the SCMC genes in different scenarios, as well as the role of gene expression profiles in the beginning of pregnancy. Besides, the co-expression network performed for *KHDC3L*, *NLRP5*, *OOEP*, and *TLE6* demonstrated their co-expression with genes related to different biological processes involved in human reproduction, such as DNA damage response and repair, embryo development, immune response, cell division, chromosome segregation, and male reproduction. Therefore, although the SCMC is confirmedly present in oocytes and early embryos, the components of this complex may exert different reproductive roles in different scenarios and may be considered in future studies aiming to understand reproductive failures of both embryonic, maternal and/or paternal origin.

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Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicial to the impartiality of the reported research.

Author Contributions

MKR contributed to the study conception, acquisition of data, and data analysis, and drafted the manuscript. TWK supervised the entire study, including conception, analysis, interpretation of data, and revisions. LRF participated in the interpretation of data and manuscript revisions. MTVS contributed to the study conception, interpretation of data, and revisions to the manuscript. All authors read and approved the final manuscript.

References

Ashary N, Tiwari A and Modi D (2018) Embryo implantation: War in times of love. Endocrinology 159:1188–1198.

Barrett T, Wilhite S, Ledoux P, Evangelista C, Kim I, Tomashevsky M, Marshall K, Phillippy K, Sherman P, Holko M *et al.* (2013) NCBI GEO: Archive for functional genomics data sets--update. Nucleic Acids Res 41:991–995.

Bebbere D, Albertini DF, Coticchio G, Borini A and Ledda S (2021) The subcortical maternal complex: Emerging roles and novel perspectives. Mol Hum Reprod 27:gaab043.

Carson SA and Kallen AN (2021) Diagnosis and management of infertility: A Review. JAMA 326:65–76.

Conti M and Franciosi F (2018) Acquisition of oocyte competence to develop as an embryo: Integrated nuclear and cytoplasmic events. Hum Reprod Update 24:245–266.

Demond H, Anvar Z, Jahromi BN, Sparago A, Verma A, Davari M, Calzari L, Russo S, Jahromi MA, Monk D *et al.* (2019) A KHDC3L mutation resulting in recurrent hydatidiform mole causes genome-wide DNA methylation loss in oocytes and persistent imprinting defects post-fertilisation. Genome Med 11:84–97.

Dimitriadis E, Menkhorst E, Saito S, Kutteh WH and Brosens JJ (2020) Recurrent pregnancy loss. Nat Rev Dis Prim 6:98-116.

Docherty LE, Rezwan FI, Poole RL, Turner CLS, Kivuva E, Maher ER, Smithson SF, Hamilton-Shield JP, Patalan M, Gizewska M *et al.* (2015) Mutations in NLRP5 are associated with reproductive wastage and multilocus imprinting disorders in humans. Nat Commun 6:8086.

Edgar R, Domrachev M and Lash A (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 30:207–210.

Eggermann T, Kadgien G, Begemann M and Elbracht M (2021) Biallelic PADI6 variants cause multilocus imprinting disturbances and miscarriages in the same family. Eur J Hum Genet 29:575–580.

Franasiak JM, Alecsandru D, Forman EJ, Gemmell LC, Goldberg JM, Llarena N, Margolis C, Laven J, Schoenmakers S and Seli E (2021) A review of the pathophysiology of recurrent implantation failure. Fertil Steril 116:1436–1448.

Gautier L, Cope L, Bolstad BM and Irizarry RA (2004) Affy – Analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 20:307–315.

Huang Z, Du G, Huang X, Han L, Han X, Xu B, Zhang Y, Yu M, Qin Y, Xia Y *et al.* (2018) The enhancer RNA lnc-SLC4A1-1 epigenetically regulates unexplained recurrent pregnancy loss (URPL) by activating CXCL8 and NF-kB pathway. EBioMedicine 38:162–170.

Jalili V, Afgan E, Gu Q, Clements D, Blankenberg D, Goecks J, Taylor J and Nekrutenko A (2020) The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2020 update. Nucleic Acids Res 48:W395–W402

Ji M, Shi X, Xiang Y, Cui Q and Zhao J (2019) NLRP7 and KHDC3L variants in Chinese patients with recurrent hydatidiform moles. Jpn J Clin Oncol 49:620–627.

Kim D, Paggi J, Park C, Bennett C and Salzberg S (2019) Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nat Biotechnol 37:907–915.

Kim S-M and Kim J-S (2017) A review of mechanisms of implantation. Dev Reprod 21:351-359.

Knöfler M, Haider S, Saleh L, Pollheimer J, Gamage TKJB and James J (2019) Human placenta and trophoblast development: Key molecular mechanisms and model systems. Cell Mol Life Sci 76:3479–3496.

Lédée N, Munaut C, Aubert J, Sérazin V, Rahmati M, Chaouat G, Sandra O and Foidart JM (2011) Specific and extensive endometrial deregulation is present before conception in IVF/ICSI repeated implantation failures (IF) or recurrent miscarriages. J Pathol 225:554–564.

Li L, Baibakov B and Dean J (2008a) A subcortical maternal complex essential for preimplantation mouse embryogenesis. Dev Cell 15:416–425

Li L, Baibakov B and Dean J (2008b) A subcortical maternal complex essential for preimplantation mouse embryogenesis. Dev Cell 15:416–425.

Liao Y, Smyth GK and Shi W (2014) featureCounts: An efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30:923–930.

Lu X, Gao Z, Qin D and Li L (2017) A maternal functional module in the mammalian oocyte-to-embryo transition. Trends Mol Med 23:1014–1023.

Montojo J, Zuberi K, Rodriguez H, Kazi F, Wright G, Donaldson S, Morris Q and Bader G (2010) GeneMANIA Cytoscape plugin: Fast gene function predictions on the desktop. Bioinformatics 26:2927–2928.

Mu J, Wang W, Chen B, Wu L, Li B, Mao X, Zhang Z, Fu J, Kuang Y, Sun X *et al.* (2019) Mutations in NLRP2 and NLRP5 cause female infertility characterised by early embryonic arrest. J Med Genet 56:471–480.

Munro SK, Farquhar CM, Mitchell MD and Ponnampalam AP (2010) Epigenetic regulation of endometrium during the menstrual cycle. Mol Hum Reprod 16:297–310.

Platnich JM and Muruve DA (2019) NOD-like receptors and inflammasomes: A review of their canonical and non-canonical signaling pathways. Arch Biochem Biophys 670:4–14.

Ritchie M, Phipson B, Wu D, Hu Y, Law C, Shi W and Smyth G (2015) *Limma* powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 43:47–59.

Robinson MD, McCarthy DJ and Smyth GK (2010) edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139–140.

Rockenbach MK, Fraga LR, Kowalski TW and Sanseverino MTV (2023) Expression profiles of meiotic genes in male vs. female gonads and gametes: Insights into fertility issues. Front Genet 14:1125097.

Shahbazi MN and Zernicka-Goetz M (2018) Deconstructing and reconstructing the mouse and human early embryo. Nat Cell Biol 20:878–887.

Sparago A, Verma A, Patricelli MG, Pignata L, Russo S, Calzari L, De Francesco N, Del Prete R, Palumbo O, Carella M *et al.* (2019) The phenotypic variations of multi-locus imprinting disturbances associated with maternal-effect variants of NLRP5 range from overt imprinting disorder to apparently healthy phenotype. Clin Epigenetics 11:190.

Vander Borght M and Wyns C (2018) Fertility and infertility: Definition and epidemiology. Clin Biochem 62:2–10.

Woods L, Perez-Garcia V and Hemberger M (2018) Regulation of placental development and its impact on fetal growth-new insights from mouse models. Front Endocrinol (Lausanne) 9:570.

Xu R, Li C, Liu X and Gao S (2021) Insights into epigenetic patterns in mammalian early embryos. Protein Cell 12:7–28.

Xu Y, Qian Y, Liu Y, Wang Q, Wang R, Zhou Y, Zhang C, Pang Z, Ye H, Xue S *et al.* (2020) A novel homozygous variant in NLRP5 is associate with human early embryonic arrest in a consanguineous Chinese family. Clin Genet 98:69–73.

Yu M, Du G, Xu Q, Huang Z, Huang X, Qin Y, Han L, Fan Y, Zhang Y, Han X *et al.* (2018) Integrated analysis of DNA methylome and transcriptome identified CREB5 as a novel risk gene contributing to recurrent pregnancy loss. EBioMedicine 35:334–344.

Yu X, Yi Z, Gao Z, Qin D, Zhai Y, Chen X, Ou-Yang Y, Wang Z, Zheng P, Zhu M *et al.* (2014) The subcortical maternal complex controls symmetric division of mouse zygotes by regulating F-actin dynamics. Nat Commun 5:4887–4898.

Zhang W, Chen Z, Zhang D, Zhao B, Liu L, Xie Z, Yao Y and Zheng P (2019) KHDC3L mutation causes recurrent pregnancy loss by inducing genomic instability of human early embryonic cells. PLoS Biol 17:e3000468.

Zhao B, Zhang W, Cun Y, Li J, Liu Y, Gao J, Zhu H, Zhou H, Zhang R and Zheng P (2018) Mouse embryonic stem cells have increased capacity for replication fork restart driven by the specific Filia-Floped protein complex. Cell Res 28:69–89.

Zhao Z, Li Y, Cao J, Fang H, Zhang L and Yang L (2022) Early pregnancy modulates expression of the nod-like receptor family in lymph nodes of ewes. Animals (Basel) 12:3285.

Zhu K, Yan L, Zhang X, Lu X, Wang T, Yan J, Liu X, Qiao J and Li L (2014) Identification of a human subcortical maternal complex. Mol Hum Reprod 21:320–329.

Supplementary material

The following online material is available for this article:

Table S1 – Datasets selected for the differential gene expression analysis.

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