



Article

MicroRNAs in the Pathogenesis of Preeclampsia—A Case-Control In Silico Analysis

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Abstract: Preeclampsia (PE) occurs in 5% to 7% of all pregnancies, and the PE that results from abnormal placentation acts as a primary cause of maternal and neonatal morbidity and mortality. The objective of this secondary analysis was to elucidate the pathogenesis of PE by probing protein–protein interactions from in silico analysis of transcriptomes between PE and normal placenta from Gene Expression Omnibus (GSE149812). The pathogenesis of PE is apparently determined by associations of miRNA molecules and their target genes and the degree of changes in their expressions with irregularities in the functions of hemostasis, vascular systems, and inflammatory processes at the fetal–maternal interface. These irregularities ultimately lead to impaired placental growth and hypoxic injuries, generally manifesting as placental insufficiency. These differentially expressed miRNAs or genes in placental tissue and/or in blood can serve as novel diagnostic and therapeutic biomarkers.

Keywords: preeclampsia; pathogenesis; transcriptome; miRNA; bioinformatics



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1. Introduction

Preeclampsia (PE) is a complication of pregnancy with symptoms of high blood pressure, proteinuria, or other signs of organ damage, and occurs in 5% to 7% of pregnancies. It is one of the leading causes of maternal morbidity. Annually, PE causes over 70,000 maternal deaths and 500,000 fetal deaths worldwide [1]. Risk factors for PE include first pregnancy; previous occurrence of PE; history of hypertension; chronic kidney disease; history of thrombophilia; pregnancy from in vitro fertilization; family history of PE; type 1 or type 2 diabetes; a body mass index (BMI) of ≥ 35 kg/m²; advanced maternal age (≥ 40 years); and prolonged interval since last pregnancy [2].

Genetic factors were associated with the occurrence of PE [3]. In a previous study by Moufarrej et al. (2022), marked cell-free RNA (cfRNA) transcriptomic changes were observed between normotensive and preeclamptic mothers early in gestation, well before the onset of PE symptoms [4]. Furthermore, their study validated a panel of 18 genes using cfRNA expression to identify the mothers at risk of preeclampsia at 5 to 16 weeks of gestation, long before the manifestation of clinical symptoms [4].

Preeclampsia that originates from abnormal placentation primarily causes maternal and neonatal morbidity and mortality [5,6]. However, the cause of the abnormal development of the placenta remains poorly understood [7,8]. Genes were found to be differentially expressed between PE and normal placenta tissues and were associated with PE pathogenesis [5]. Hence, studies have been focused on the genetic signature of the placenta from preeclampsia.

Recent advances in high-throughput in silico techniques portray experimental data into exemplified biological networks. Exploring these biological networks can disclose the role of individual proteins, protein–protein interactions (PPIs), and corresponding biological functions. This study intended to use the transcriptomic profiling of mRNA in preeclamptic (PE) and normal placentae from Gene Expression Omnibus (GSE149812) for

further in silico analysis to elucidate the involvement of placenta-specific miRNA in the pathogenesis of PE.

2. Materials and Methods

In this study, differentially expressed PE-associated genes were identified from transcriptome data of PE and normal placenta samples. The gene expression data (profiled by microarray) and clinical characteristics were downloaded from the Gene Expression Omnibus (GSE149812; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE149812>; Accessed 7 July 2023). In the primary data, the description included patients' clinical characteristics, tissue collection, RNA extraction, and microarray analysis methods. An excerpt of the description is provided below in Sections 2.1 and 2.2.

2.1. Patients and Tissue Collection

Placental biopsies were obtained during cesarean section from both normotensive patients ($n = 3$) and those with preeclampsia ($n = 3$) (early onset type of PE; <31 weeks of gestation). All patients involved in this study were recruited from the Department of Obstetrics and Gynecology, the Third Xiangya Hospital, Central South University, Hunan, China. Pieces of villous tissue ($0.5 \times 0.5 \times 0.5 \text{ cm}^3$), approximately 2 cm beside the umbilical cord insertion, from the middle layer of the placenta midway between the maternal and fetal surfaces from different areas, were excised, excluding sites of hemorrhage, infarction, and fibrin deposition. Tissues were immediately placed in 1.0 mL RNASTore Reagent (CWbiotech Company, Taizhou, China), and then stored at -80°C until use.

2.2. RNA Extraction and Microarray Analysis

Total RNA was extracted using TRIzol following the manufacturer's instructions. Cyanine-3 (Cy3) labeled complementary RNA (cRNA) was prepared from 0.5 μg RNA using the One-Color Low RNA Input Linear Amplification PLUS kit (Agilent Tech. Inc., Santa Clara, CA, USA), followed by RNAeasy column purification (Qiagen Inc., Valencia, CA, USA). The cRNA yield was checked by an ND-1000 Spectrophotometer. Then, 1.5 μg of Cy3-labeled cRNA (specific activity $> 10.0 \text{ pmol Cy3}/\mu\text{g cRNA}$) was fragmented at 60°C for 30 min in a reaction volume of 250 μL containing $1 \times$ Agilent fragmentation buffer and $2 \times$ Agilent blocking buffer. On completion of the fragmentation reaction, 250 μL of $2 \times$ Agilent hybridization buffer was added to the fragmentation mixture and hybridized to Phalanx Human OneArray ver. 6 Release 1 for 17 h at 65°C in a rotating Agilent hybridization oven. After hybridization, microarray slides were washed for 1 min at room temperature with GE wash buffer 1 (Agilent) and 1 min with 37°C GE wash buffer 2 (Agilent) and then dried immediately by brief centrifugation. Slides were scanned immediately after washing on an Agilent DNA Microarray Scanner (G2505B) using one color scan setting for $1 \times 44\text{k}$ array slides (scan area of $61 \times 21.6 \text{ mm}^2$; scan resolution of $10 \mu\text{m}$; dye channel set to Green, and Green PMT was set to 100%). The scanned images were analyzed using Feature Extraction Software 9.1 (Agilent).

2.3. Data Processing

The data were analyzed with GEO2R to identify genes that are differentially expressed between the two groups. GEO2R uses DESeq2, which is an R package for identifying differentially expressed genes from RNA-seq data [9,10] using negative binomial generalized linear models, which are suitable for studies with few replicates [10]. A 5-fold relative difference ($p \leq 0.05$) was used as a cut-off for the selection of differentially expressed (upregulated and downregulated) genes for further in silico analysis.

2.4. In Silico Analysis

2.4.1. Prediction and Analysis of Differentially Expressed Genes

The updated miRNet (<http://www.mirnet.ca/>, accessed on 1 July 2023) platform was used [11] to perform interaction analysis, separately, for upregulated and downregulated

genes. The degree (defined by the number of connections a node has to other nodes) and betweenness (defined by the number of connections occurring upon a node) of miRNAs and genes in the network were determined.

2.4.2. Gene Ontology and Functional Annotation Analysis of Genes with the Highest Degree and Betweenness Centrality

The top 20 up- and downregulated genes with the highest degree and betweenness centrality were selected, and their tissue expression, associated interacting genes (up to 6 genes; <http://stringdb.org/>; accessed on 6 July 2023), and single-cell normalized expression (<https://www.proteinatlas.org/>; accessed on 6 July 2023) were investigated.

2.4.3. Gene Ontology Enrichment and KEGG Pathway Analysis

All differentially expressed genes from the network were retrieved to recognize PPIs. The PPI network was created using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) online database (<http://stringdb.org/>; accessed on 1 July 2023) separately for upregulated and downregulated genes [12]. Gene Ontology (GO) functional annotation for biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were also performed. A *p*-value of <0.05 was regarded as statistically significant.

2.4.4. Identification and Analysis of Hub Gene

The PPI networks for upregulated and downregulated genes from the STRING database were exported to Cytoscape software (version 3.10) [13]. The hub genes were selected as the top 20 nodes of the PPI network using the Maximal Clique Centrality (MCC) method [14], which has a better performance on the precision of predicting top essential proteins. Further analysis was performed using ClueGO [15] to integrate GO terms as well as KEGG pathways and create a functionally nested or organized GO/pathway term (*k*-score = 3). This task compares one set of genes or two lists of genes and comprehensively visualizes functionally grouped terms [15].

2.4.5. Gene Ontology and Functional Annotation Analysis of Hub Genes

The hub genes and their roles, tissue expression, and protein–protein interactions (up to 6 closely related genes) for differentially expressed genes in women with PE from STRING (<http://stringdb.org/>; accessed on 4 July 2023) and human protein atlas (<https://www.proteinatlas.org/>; accessed on 4 July 2023) were investigated. To substantiate their presence, tissue expression and organelle localization were presented.

2.4.6. Comparison of miRNAs of Different Types of Preeclampsia

For comparison of different types (early- vs. late-onset; mild vs. severe) of preeclampsia, we selected DE genes in early-onset severe preeclampsia, late-onset severe preeclampsia, and late-onset mild preeclampsia from RNA-seq on 65 high-quality placenta samples that included 33 from 30 PE patients and 32 from 30 control subjects reported by Ren et al., 2021 [16]. These DE gene sets representing different types of PE were subjected to gene-miRNA interaction analysis.

3. Results

The transcriptomic (mRNA) profiling between PE and normal placenta tissues from Gene Expression Omnibus (GSE149812) recognized 28,254 genes (Supplementary File S1). There were 79 and 60 up- and downregulated genes, respectively (Supplementary File S1). Of those differentially expressed genes, 52 and 42 up- and downregulated genes, respectively, were at a 5-fold difference ($p \leq 0.05$; Supplementary File S2). The gene-miRNA interaction network analysis revealed the involvement of 45 upregulated and 32 downregulated genes.

Table 1. Top 20 upregulated genes in the placenta with high degree and betweenness centrality in preeclamptic compared to normotensive women.

High Degree Centrality				High Betweenness Centrality			
#	ID	Degree	Betweenness	#	ID	Degree	Betweenness
1	TGFBR1	129	62,386.63603	1	DUSP4	124	66,214.0901
2	DUSP4	124	66,214.0901	2	TGFBR1	129	62,386.63603
3	TMCC1	122	60,207.54204	3	TMCC1	122	60,207.54204
4	EMP1	113	59,488.02209	4	EMP1	113	59,488.02209
5	BHLHE40	111	53,832.72771	5	BHLHE40	111	53,832.72771
6	PDS5A	105	46,221.20935	6	PDS5A	105	46,221.20935
7	PPIG	96	41,670.73543	7	PPIG	96	41,670.73543
8	IPPK	70	28,805.24642	8	SFT2D3	61	32,179.26096
9	STIP1	65	27,238.03764	9	IPPK	70	28,805.24642
10	DESI2	62	17,175.83835	10	STIP1	65	27,238.03764
11	SFT2D3	61	32,179.26096	11	PHLDA2	52	26,712.22297
12	SORL1	59	21,899.50057	12	FLT1	57	23,913.42422
13	FLT1	57	23,913.42422	13	MRPL49	44	23,540.21993
14	PHLDA2	52	26,712.22297	14	GJB7	40	22,523.85633
15	MRPL49	44	23,540.21993	15	SORL1	59	21,899.50057
16	GJB7	40	22,523.85633	16	TMEM54	36	18,180.58635
17	TMEM54	36	18,180.58635	17	DESI2	62	17,175.83835
18	DHFR	34	10,104.03447	18	SSX5	22	13,882.13629
19	RASSF6	32	13,330.8966	19	RASSF6	32	13,330.8966
20	HLA-DQA1	28	12,741.03278	20	HLA-DQA1	28	12,741.03278

All genes that showed high degree centrality also had high betweenness centrality except the gene in bold letters.

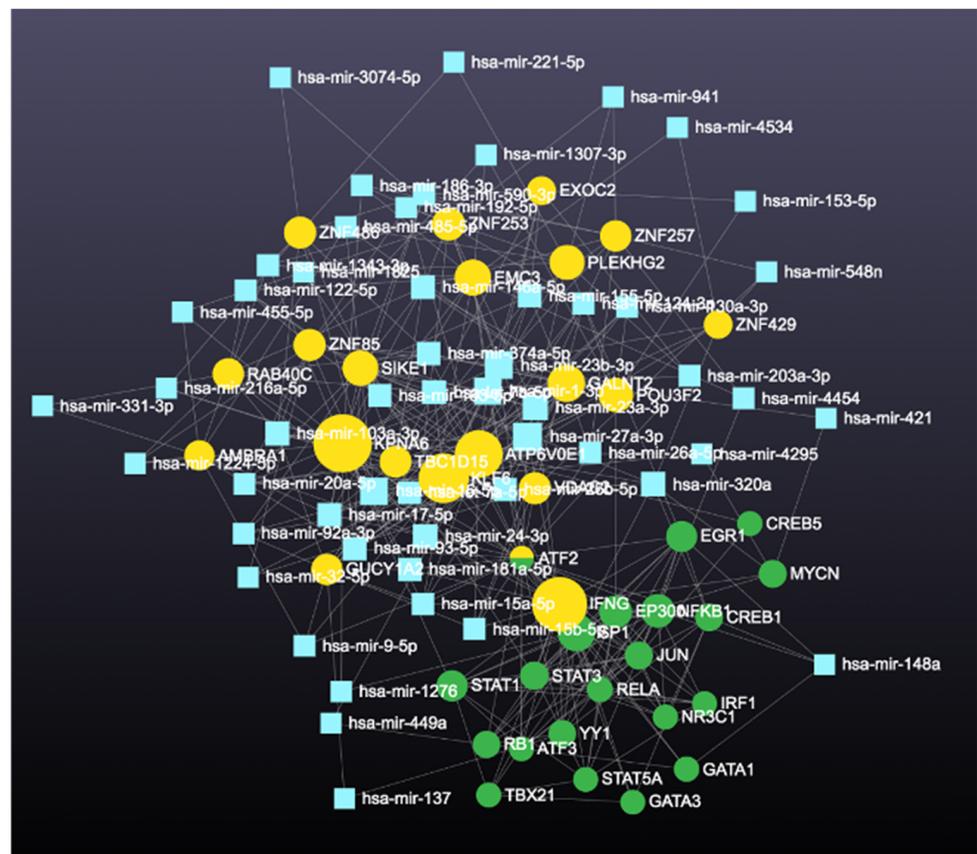


Figure 4. Gene-miRNA interaction network for the top 20 downregulated genes. Green circles denote genes. Yellow circles denote transcription factors. Size indicates significance. Blue squares denote miRNAs.

Table 2. Top 20 downregulated genes in the placenta with high degree and betweenness centrality in preeclamptic compared to normotensive women.

High Degree Centrality				High Betweenness Centrality			
#	ID	Degree	Betweenness	#	ID	Degree	Betweenness
1	KPNA6	223	161,133.371	1	KPNA6	223	161,133.371
2	ATP6V0E1	152	90,977.3419	2	ATP6V0E1	152	90,977.34186
3	KLF6	129	75,754.0689	3	KLF6	129	75,754.06887
4	SIKE1	118	60,992.2768	4	PLEKHG2	112	71,728.72334
5	PLEKHG2	112	71,728.7233	5	ZNF85	98	67,140.10746
6	ZNF85	98	67,140.1075	6	SIKE1	118	60,992.27675
7	EMC3	92	53,114.7673	7	EMC3	92	53,114.76726
8	GALNT2	83	38,016.4527	8	VDAC2	69	52,426.38509
9	TBC1D15	83	48,514.111	9	TBC1D15	83	48,514.11101
10	ATF2	81	35,905.8297	10	GALNT2	83	38,016.45269
11	VDAC2	69	52,426.3851	11	ATF2	81	35,905.8297
12	AMBRA1	55	27,918.5407	12	IFNG	41	32,732.16167
13	RAB40C	51	23,353.295	13	AMBRA1	55	27,918.54066
14	ZNF257	51	27,233.8438	14	ZNF486	49	27,772.96946
15	ZNF429	51	24,069.767	15	EXOC2	49	27,644.24328
16	EXOC2	49	27,644.2433	16	ZNF257	51	27,233.84385
17	ZNF486	49	27,772.9695	17	GUCY1A2	47	25,416.96916
18	ZNF253	47	23,149.4803	18	ZNF429	51	24,069.76705
19	GUCY1A2	47	25,416.9692	19	RAB40C	51	23,353.29504
20	POU3F2	44	22,680.8773	20	ZNF253	47	23,149.48028

All genes that showed high degree centrality also had high betweenness centrality except the genes in bold letters.

Table 3. Top 20 upregulated genes (in the placenta with a high degree and betweenness centrality) and their tissue and single-cell expressions, associated genes, and functions.

Gene	Tissue Expression	Single-Cell Normalized Expression (nTPM)	Associated Genes	Functions
TGFBRI	Ovary, uterus placenta	Cyto 22.1; Syncytio: 18.4; extravillous: 7.3; Endometrium 21.2	FKBP1A, TGFB1, TGFB3, TGFB2, SMAD7	Regulates cellular process: proliferation, maturation, differentiation, motility, and apoptosis
DUSP4	Ovary, uterus placenta	Cyto 3.0; Syncytio: 24.9; extravillous: 48.8; Endometrium 13.7	MAPK1, MAPK3, MAPK7, MAPK8, MAPK9	Regulates cell proliferation and differentiation
TMCC1	Ovary, uterus placenta	Cyto: 10.4; Syncytio: 27.3; extravillous: 0.6; Endometrium 14.2	PLEC, RSP10, RSP10-NUDT3, RSP12, RSP18A, RSP19	Regulates endosome fission; endosome membrane tubulation; and membrane fission
EMP1	Ovary, uterus placenta	Cyto: 0.7; Syncytio: 0.7; extravillous: 0.6; Endometrium 161.6	CCL4, LPAR6, LAPTM4B, PMP22, SMIM3	Regulates cell proliferation and migration
BHLHE40	Ovary, uterus placenta	Cyto: 31.8; Syncytio: 165.5; extravillous: 94.7; Endometrium 68.0	BTRC, HDAC1, RXRA, TP53, SMAP2	Regulates circadian rhythm and cell differentiation
PDS5A	Ovary, uterus placenta	Cyto: 32.6; Syncytio: 37.7; extravillous: 39.0; Endometrium 47.3	RAD21, SMC1A, SMC3, STAG2, WAPAL	Regulates chromatid cohesion during mitosis
PPIG	Ovary, uterus placenta	Cyto: 186.3; Syncytio: 241.4; extravillous: 200.9; Endometrium 146.2	BUD31, PCBP1, PRPF8, PRPF19, SNW1	Regulates folding, transport, and assembly of proteins, and pre-mRNA splicing
IPPK	Ovary, uterus placenta	Cyto: 10.9; Syncytio: 23.7; extravillous: 14.8; Endometrium 4.6	EPB41L4A, FRMD5, LPAR1, MPKAPK5, VRK1	Regulates DNA repair, endocytosis, and mRNA export
STIP1	Ovary, uterus placenta	Cyto: 127.7; Syncytio: 210.3; extravillous: 143.4; Endometrium 48.8	HSP8, HSPA1A, HSP90AA1, HSP90AB1, PTGES3	Regulates heat shock proteins
DESI2	Ovary, uterus placenta	Cyto: 30.7; Syncytio: 43.8; extravillous: 42.9; Endometrium 39.9	DDX5, E2F8, NPML, NUP107, RPA1, UBE2I	Regulates protein deubiquitination
SFT2D3	Ovary, uterus placenta	Cyto: 4.3; Syncytio: 3.0; extravillous: 4.2; Endometrium 8.9	ADHFE1, ADACC, COG1, PSAT1, TMEM24, TSGA13	Regulates protein transport and vesicle-mediated transport
SORL1	Ovary, uterus placenta	Cyto: 0.2; Syncytio: 0.4; extravillous: 2444.5; Endometrium 2.9	APP, APOE, CGA1, LRPAP1, VPS35	Regulates protein transport
FLT1	Ovary, uterus placenta	Cyto: 182.7; Syncytio: 10,058.3; extravillous: 980.8; Endometrium 1.4	KDR, PGF, PTPN11, VEGFA, VEGFB	Regulates angiogenesis and vasculogenesis
PHLDA2	Ovary, uterus placenta	Cyto: 4565.5; Syncytio: 365.0; extravillous: 336.1; Endometrium 27.9	RANBP9, SUCO, SRC	Regulates fetal and placental growth
MRPL49	Ovary, uterus placenta	Cyto: 63.8; Syncytio: 119.5; extravillous: 49.1; Endometrium 11.3	COX15, TIMM10, METTL18, NXF1, FBXW11	Regulates protein metabolism and mitochondrial translation
GJB7	Ovary, uterus placenta	Cyto: 10.7; Syncytio: 8.9; extravillous: 4.7; Endometrium 0.7	ARVCF, FYN, PAG1, PPP2R5E, ULBP2	Regulates gap junction trafficking and vesicle-mediated transport
TMEM54	Ovary, uterus placenta	Cyto: 48.2; Syncytio: 64.7; extravillous: 169.6; Endometrium 16.9	CREB3, CDK2, HDAC1, LMNA, PEX19, RARA	Regulates membrane function
DHFR	Ovary, uterus placenta	Cyto: 34.5; Syncytio: 12.1; extravillous: 40.1; Endometrium 6.9	FOX1, HSPD1, MDM2, FKBP1A, TP53,	Regulates folate metabolism and glycine and purine synthesis
RASSF6	Ovary, uterus placenta	Cyto: 54.5; Syncytio: 48.9; extravillous: 24.8; Endometrium 2.0	AMY1A, DLG1, KDM3A, HECTD1, SAV1, STK4	Regulates cell cycle arrest and apoptosis
HLA-DQA1	Ovary, uterus placenta	Cyto: 6.9; Syncytio: 4.8; extravillous: 10.7; Endometrium 33.4	CD74, HLA-DQB1, KCNJ8, ST7, SLC38A9, TMEM214	Regulates immune function
SSX5	Ovary, uterus placenta	Cyto: 0; Syncytio: 0; extravillous: 0; Endometrium 0	AGTRAP, PCBD2, NFE2, SSX2, ZSCAN1	Regulates immune function

Cyto—Cytotrophoblast; Syncytio—syncytiotrophoblast; extravillous—extravillous trophoblast; Endometrium—endometrial stromal cells.

Table 4. Top 20 downregulated genes (in the placenta with a high degree and betweenness centrality) and their tissue and single-cell expressions, associated genes, and functions.

Gene	Tissue Expression	Single-Cell Normalized Expression (nTPM)	Associated Genes	Functions
KPNA6	Ovary, uterus placenta	Cyto 41.2; Syncytio: 138.3; extravillous: 37.6; Endometrium 39.7	HDAC1, KPNB1, LMNA, NUP50, RELB	Regulates protein transport
ATP6V0E1	Ovary, uterus placenta	Cyto 511.0; Syncytio: 985.2; extravillous: 643.8; Endometrium 199.9	ACP2, SLC7A2, CCDC115, PTPRF, TMEM199	Regulates protein transport and pH of intercellular compartments
KLF6	Ovary, uterus placenta	Cyto: 176.5; Syncytio: 217.0; extravillous: 539.4; Endometrium 616.8	HDAC3, KLF4, LCOR, RELA, SP1	Regulates cell growth
SIKE1	Ovary, uterus placenta	Cyto: 30.8; Syncytio: 38.0; extravillous: 36.2; Endometrium 34.4	PPP2R1A, PPP2CA, STRN4, STK24, STK25, TRAF3IP3	Plays inhibitory role in virus- and TLR3-triggered IRF3
PLEKHG2	Ovary, uterus placenta	Cyto: 0.7; Syncytio: 0.6; extravillous: 2.9; Endometrium 18.6	CDC42, GNB1, GNG2, RAC1, RHOA	Regulates lymphocyte chemotaxis via Rac and Cdc42 activation and actin polymerization
ZNF85	Ovary, uterus placenta	Cyto: 10.5; Syncytio: 6.1; extravillous: 15.4; Endometrium 4.0	CEP76, TRIM28	Regulates DNA templated transcription
EMC3	Ovary, uterus placenta	Cyto: 50.9; Syncytio: 91.6; extravillous: 57.7; Endometrium 50.2	EMC1, EMC2, EMC4, EMC6, MMG1	Regulates membrane insertase activity
GALNT2	Ovary, uterus placenta	Cyto: 6.9; Syncytio: 14.1; extravillous: 141.2; Endometrium 13.3	AP4M1, AP4S1, MMG1, MRPS5, ZMPSTE24	Regulates glycosylation of protein
TBC1D15	Ovary, uterus placenta	Cyto: 20.5; Syncytio: 48.2; extravillous: 16.3; Endometrium 39.6	CCDC121, CEP23, OPTN, TBC1D17, UBXN8	Regulates GTPase activator activity and mitochondrial morphology
ATF2	Ovary, uterus placenta	Cyto: 13.9; Syncytio: 6.0; extravillous: 13.5; Endometrium 28.1	FOS, JUN, MAPK8, MAPK9, MAPK14	Regulates transcription of various genes involved in apoptosis, cell growth, proliferation, inflammation, and DNA damage response
VDAC2	Ovary, uterus placenta	Cyto: 334.2; Syncytio: 399.4; extravillous: 470.9; Endometrium 107.0	COX4I1, NDUFS4, PHB, PHB2, VDAC2	Regulates oxidative metabolism, ion transport, cell apoptosis
AMBRA1	Ovary, uterus placenta	Cyto: 3.9; Syncytio: 7.3; extravillous: 2.0; Endometrium 4.8	BECN1, CUL4A, DDA1, DDB1, TCEB2	Regulates mitophagy, cell proliferation, cell cycle progression
RAB40C	Ovary, uterus placenta	Cyto: 26.0; Syncytio: 51.3; extravillous: 15.8; Endometrium 6.7	CUX2, CUX2, ENSP00000447000, RAB40B, SARNP	Regulates protein metabolism and autophagy
ZNF257	Ovary, uterus placenta	Cyto: 4.2; Syncytio: 3.0; extravillous: 5.5; Endometrium 1.4	HIST1H3A, SSRP1, CTCF, GL13, ZNF 513, ZNF710, ZNF768	Regulates DNA templated transcription, apoptosis, protein folding and assembly, and lipid binding
ZNF429	Ovary, uterus placenta	Cyto: 14.7; Syncytio: 12.7; extravillous: 11.7; Endometrium 10.3	CTCF, GL13, ZNF 513, ZNF710, ZNF768	Regulates transcription by RNA polymerase II, apoptosis, protein folding and assembly, and lipid binding
EXOC2	Ovary, uterus placenta	Cyto: 15.3; Syncytio: 13.9; extravillous: 6.6; Endometrium 6.2	EXOC3, EXOC4, EXOC5, EXOC6, EXOC7	Regulates polarized targeting of exocytic vesicles to specific docking sites on the plasma membrane
ZNF486	Ovary, uterus placenta	Cyto: 4.6; Syncytio: 1.8; extravillous: 15.7; Endometrium 6.5	CTCF, GL13, ZNF 513, ZNF710, ZNF768	Regulates DNA templated transcription, apoptosis, protein folding and assembly, and lipid binding
ZNF253	Ovary, uterus placenta	Cyto: 5.5; Syncytio: 4.5; extravillous: 3.4; Endometrium 5.2	AKR1B1, LDOC1, CTCF, ZNF 513, ZNF710	Regulates DNA templated transcription, apoptosis, protein folding and assembly, and lipid binding
GUCY1A2	Ovary, uterus placenta	Cyto: 0.1; Syncytio: 0.2; extravillous: 0.0; Endometrium 2.0	GUCY1B3, DLG1, DLG2, DLG3, DLG4	Regulates conversion of GTP to 3',5'-cyclic GMP and pyrophosphate
POU3F2	Ovary, uterus placenta	Cyto: 0.0; Syncytio: 0.0; extravillous: 0.1; Endometrium 0.1	POU4F1, POU4F2, POU4F3, SOX10, TFCEP2	Regulates neuronal differentiation and activation of CRH regulated genes
IFNG	Ovary, uterus placenta	Cyto: 0.1; Syncytio: 0.1; extravillous: 0.1; Endometrium 0.9	FOXP3, IFNGR1, IFNGR2, RUNX1, TRIM2	Regulates cellular response to viral and microbial infections

Cyto—Cytotrophoblast; Syncytio—syncytiotrophoblast; extravillous—extravillous trophoblast; Endometrium—endometrial stromal cells.

After determining the degree and betweenness, the up- and downregulated genes that were 5-fold different ($p < 0.05$) were submitted (<http://stringdb.org/>; accessed on 7 July 2023) to elucidate enrichment networks. Figure 5 shows the PPIs for the upregulated genes (78 nodes; 193 edges; PPI enrichment with $p < 1.0 \times 10^{-16}$), revealing 225 significantly enriched biological process GO terms (False Recovery Rate, $p \leq 0.05$) and 54 significant (False Recovery Rate, $p \leq 0.05$) KEGG enrichment pathways (Supplementary File S2). Figure 6 shows the PPIs for the downregulated genes (73 nodes and 293 edges, PPI enrichment p -value of $<1.0 \times 10^{-16}$), revealing 268 significantly enriched biological process GO terms (False Recovery Rate, $p \leq 0.05$) and 87 significant (False Recovery Rate, $p \leq 0.05$) KEGG enrichment pathways (Supplementary File S3). The PPI networks for the up- and downregulated genes were separately constructed using the STRING database and Cytoscape software (Version 3.9). The top-ranked 20 hub genes using the Maximal Clique Centrality (MCC) method for up- and downregulated genes were screened and are presented in Figures 7 and 8, respectively. To interpret functionally nested gene ontology and pathway annotation networks for up- and downregulated genes in the PE placenta, ClueGo nested network analysis was performed, and the results are pre-

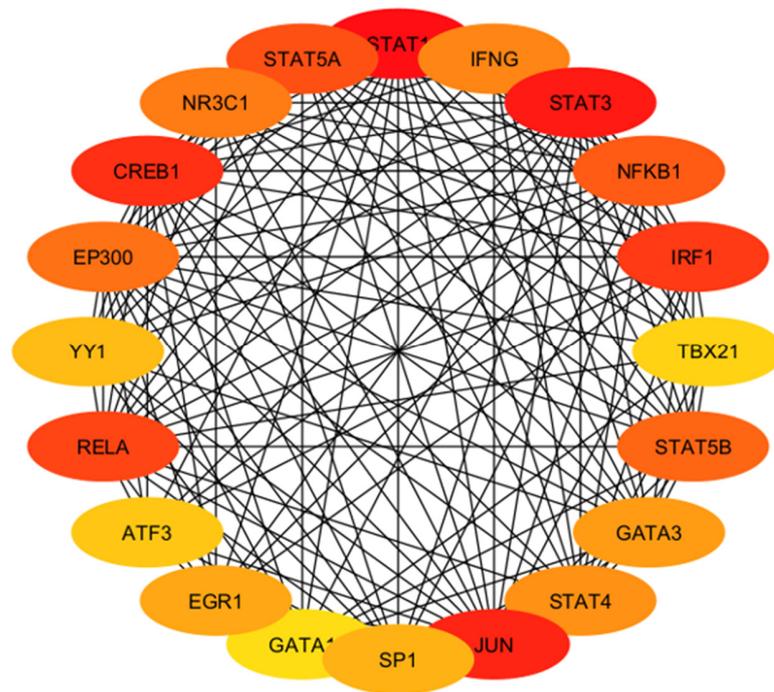


Figure 8. Interactions among hub genes (*ATF3, CREB1, EGR1, EP300, GATA1, GATA3, IFNG, IRF1, JUN, NFKB1, NR3C1, RELA, SP1, STAT1, STAT3, STAT4, STAT5A, STAT5B, TBX21, and YY1*) of downregulated genes in the protein–protein interaction network. The dark to light colors denotes high to low degrees of expression. Black lines indicate interactions between genes.

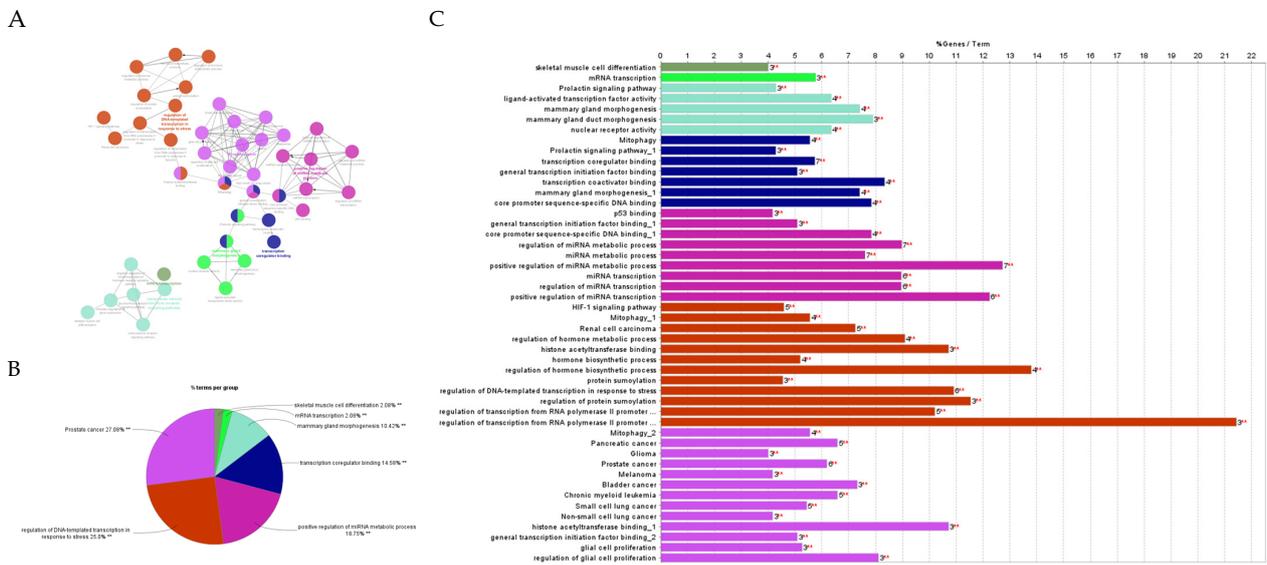


Figure 9. ClueGO analysis of upregulated genes. (A) Functionally grouped network with terms as nodes linked based on their kappa score level (≥ 0.4), where only the label of the most significant term per group is shown. The node size represents the term enrichment significance. Functionally related groups partially overlap. The grey color gradient shows the gene proportion of each cluster associated with the term. (B) Overview chart with functional groups including specific terms for upregulated genes. $** p < 0.001$. (C) GO/pathway terms specific for upregulated genes. The bars represent the number of genes (in red) associated with the terms. The percentage of genes per term is shown as a bar label.

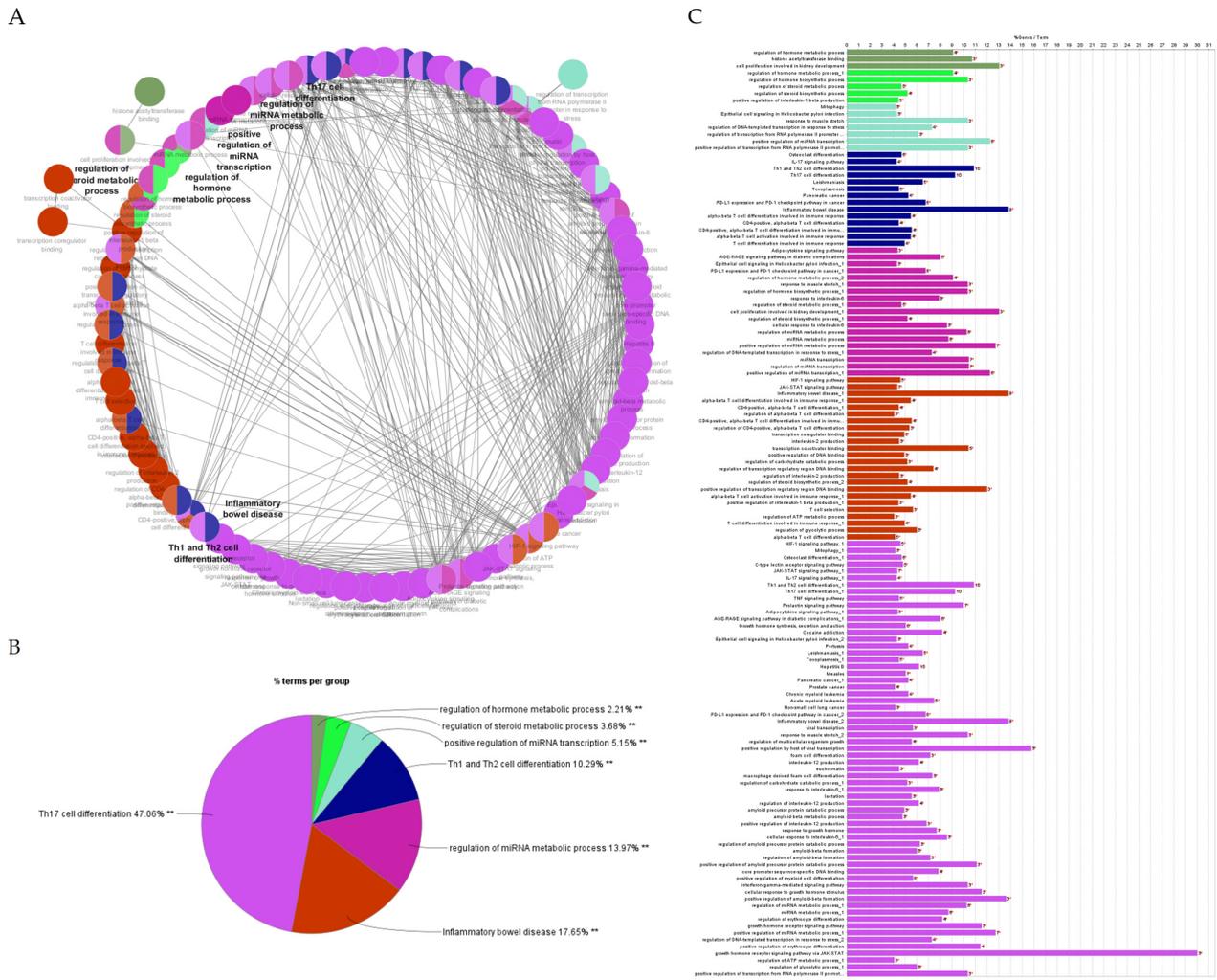


Figure 10. ClueGO analysis of downregulated genes. **(A)** Functionally grouped network with terms as nodes linked based on their kappa score level (≥ 0.4), where only the label of the most significant term per group is shown. The node size represents the term enrichment significance. Functionally related groups partially overlap. The grey color gradient shows the gene proportion of each cluster associated with the term. **(B)** Overview chart with functional groups including specific terms for upregulated genes. ****** $p < 0.001$. **(C)** GO/pathway terms specific for upregulated genes. The bars represent the number of genes associated with the terms. The percentage of genes per term is shown as a bar label.

Table 5. Top 20 upregulated hub genes and their tissue and single-cell expressions, associated genes, and functions.

Hub Gene	Tissue Expression	Single-Cell Normalized Expression (nTPM)	Associated Genes	Functions
ARNTL	Ovary, uterus placenta	Cyto 17.0; Syncytio: 6.1; extravillous: 1.3; Endometrium 13.9	CLOCK, CRY1, CRY2, NPAS2, PER2	Regulates molecular circadian rhythm, myogenesis, adipogenesis, hormone production, cell proliferation
CLOCK	Ovary, uterus placenta	Cyto 11.3; Syncytio: 6.3; extravillous: 7.0; Endometrium 35.2	ARNTL, CIPC, CRY1, CRY2, PER2	Regulates molecular circadian rhythm
NR3C1	Ovary, uterus placenta	Cyto: 48.6; Syncytio: 36.6; extravillous: 44.2; Endometrium 28.5	HSP90AA1, NCOA1, NCOA2, NCOR, SMARCA4	Regulates hypothalamic–pituitary–adrenal (HPA) axis by modulating availability of the cortisol
ETS1	Ovary, uterus placenta	Cyto: 0.1; Syncytio: 0.3; extravillous: 0.4; Endometrium 49.7	CREBBP, FOXO1, NFKB2, PAX5, RUNX1	Regulates immune cell function
EGR1	Ovary, uterus placenta	Cyto: 154.9; Syncytio: 165.7; extravillous: 106.1; Endometrium 783.3	EP300, JUNDB, JUNDD, NAB1, TP53	Regulates attachment and survival of normal cells and induces apoptosis in abnormal cells

Table 5. Cont.

Hub Gene	Tissue Expression	Single-Cell Normalized Expression (nTPM)	Associated Genes	Functions
NFKB1	Ovary, uterus placenta	Cyto: 15.2; Syncytio:13.5; extravillous: 17.3; Endometrium 60.4	NFKB1A, RELA, CHUK, IFBKB, RELB	Regulate genes
CREBBP	Ovary, uterus placenta	Cyto: 17.2; Syncytio: 32.2; extravillous: 11.1; Endometrium 52.1	CREB1, HIF1A, KMT2A, MYB, TP53	Regulates cell growth and division and prompting cells to mature and differentiate
SMARCA4	Ovary, uterus placenta	Cyto: 72.5; Syncytio: 70.3; extravillous: 62.4; Endometrium 46.0	SMARCB1, SMARCC1, SMARCC2, SMARCD1, SMARCE1	Regulates chromatin remodeling
ESR1	Ovary, uterus placenta	Cyto: 0.1; Syncytio: -; extravillous: -; Endometrium 72.4	EP300, NCOA1, NCOA2, NR2F1, NR2F2	Regulates many biological functions including growth, differentiation and function of female reproductive system, hormone binding, immune function
RELA	Ovary, uterus placenta	Cyto: 23.0; Syncytio: 47.7; extravillous: 27.7; Endometrium 24.8	BRD4, CREBBPEP300, NFKB1, NFKB1A	Regulate genes involved in apoptosis, inflammation, the immune response, and proliferation
CREB1	Ovary, uterus placenta	Cyto: 30.1; Syncytio: 18.7; extravillous: 25.9; Endometrium 37.8	CREBBP, CRTC2, EP300, RPS6KA5, TP53	Regulates proliferation, migration, and invasion of cells
VDR	Ovary, uterus placenta	Cyto: 0.1; Syncytio: 0.2; extravillous: 0.1; Endometrium 0.5	NCOA1, NCOA2, NCOA3, MED1, RXRA	Induces a surge of cell signaling to maintain healthy Ca ²⁺ levels that serve to regulate several biological functions
TP53	Ovary, uterus placenta	Cyto: 39.7; Syncytio: 20.4; extravillous: 40.6; Endometrium 28.3	CREBBP, EP300, MDM2, MDM4, RPZ27A	Regulates cell division and apoptosis
EPAS1	Ovary, uterus placenta	Cyto: 118.5; Syncytio: 365.0; extravillous: 336.1; Endometrium 31.3	ARNT, EGLN1, VHL, TCEB1, TCEB2	Regulates cell division, angiogenesis, adaptation to changing oxygen level
ARNT	Ovary, uterus placenta	Cyto: 24.1; Syncytio: 32.4; extravillous: 40.3; Endometrium 21.8	AHR, EPAS1, HIF1A, NPAS3, SIM2	Regulates placentation
VHL	Ovary, uterus placenta	Cyto: 35.3; Syncytio: 34.0; extravillous: 35.0; Endometrium 37.8	EPAS1, CUL2, HIF1A, TCEB1, TCEB2	Regulates cell growth and division
SP1	Ovary, uterus placenta	Cyto: 16.3; Syncytio: 22.4; extravillous: 17.0; Endometrium 22.3	EP300, ESR1, HDAC1, HDAC2, TP53	Regulates cell cycle, hormonal activation, apoptosis, and angiogenesis
E2F1	Ovary, uterus placenta	Cyto: 5.3; Syncytio: 2.1; extravillous: 8.8; Endometrium 1.0	CCNA2, DP2, RB1, RBL1, TFDP1	Regulates cell cycle progression, DNA repair, apoptosis
TFDP1	Ovary, uterus placenta	Cyto: 85.2; Syncytio: 60.0; extravillous: 123.9; Endometrium 27.1	E2F1, E2F4, E2F5, E2F6, RB1	Regulates cell cycle progression
RB1	Ovary, uterus placenta	Cyto: 6.9; Syncytio: 4.8; extravillous: 10.7; Endometrium 33.4	CCND1, CDK4, DNMT1, E2F1, TFDP1	Regulates cell growth and division

Cyto—Cytotrophoblast; Syncytio—syncytiotrophoblast; extravillous—extravillous trophoblast; Endometrium—endometrial stromal cells.

Table 6. Top 20 downregulated hub genes and their tissue and single-cell expressions, associated genes, and functions.

Hub Gene	Tissue Expression	Single-Cell Normalized Expression (nTPM)	Associated Genes	Functions
IFNG	Ovary, uterus placenta	Endometrium 0.9	IFNGR1, IFNGR2, FOXP3, RUNX1, TRIM28	Regulates cell differentiation, activation, expansion, homeostasis, and survival
STAT3	Ovary, uterus placenta	Cyto 27.3; Syncytio: 35.9; extravillous: 49.3; Endometrium 194.6	BMX, EGFR, JK1, MAPK1, PIAS3	Controls cell proliferation, migration, apoptosis
NFKB1	Ovary, uterus placenta	Cyto: 15.2; Syncytio:13.5; extravillous: 17.3; Endometrium 60.4	NFKB1A, RELA, CHUK, IFBKB, RELB	Regulate genes
IRF1	Ovary, uterus placenta	Cyto: 25.0; Syncytio: 9.4; extravillous: 46.9; Endometrium 179.7	IRF8, STUB1, STAT1, EP300, KAT2B	Regulate innate and adaptive immune responses
TBX21	Ovary, uterus placenta	-	CREBBP, EP300, GATA3, SPI1, UBC, TBX21	Regulates development of naive T lymphocytes
STAT5B	Ovary, uterus placenta	Cyto: 8.0; Syncytio: 13.8; extravillous: 5.9; Endometrium 20.5	EGFR, INSR, JAK1, JAK2, JAK3	Regulates formation of tissues and organs; maintains immune homeostasis
GATA3	Ovary, uterus placenta	Cyto: 329.4; Syncytio: 1237.7; extravillous: 843.6; Endometrium 0.4	HDAC1, HDAC2, HDAC3, LMO1, TAL1	Regulates cell maturation with proliferation arrest and cell survival
STAT4	Ovary, uterus placenta	Cyto: 0.4; Syncytio: 0.4; extravillous: 3.4; Endometrium 0.4	JUN, IL12RB2, PIAS2, STAT1, ZNF467	Regulates innate and adaptive immune responses
JUN	Ovary, uterus placenta	Cyto: 666.6; Syncytio: 405.9; extravillous: 61.9; Endometrium 2873.0	ATF2, FOS, MAPK8, MAPK9, MAPK10	Cell proliferation, apoptosis and survival, and tissue morphogenesis
SP1	Ovary, uterus placenta	Cyto: 16.3; Syncytio: 22.4; extravillous: 17.0; Endometrium 22.3	EP300, ESR1, HDAC1, HDAC2, TP53	Regulates cell cycle, hormonal activation, apoptosis, and angiogenesis
GATA1	Ovary, uterus placenta	-	BRD3, FLJ11, LMO2, TAL1, ZFPM1	Regulates development of multipotential progenitors and hematopoietic stem cells
EGR1	Ovary, uterus placenta	Cyto: 154.9; Syncytio: 165.7; extravillous: 106.1; Endometrium 783.3	EP300, JUNDB, JUNDD, NAB1, TP53	Regulates attachment and survival of normal cells and induces apoptosis in abnormal cells
ATF3	Ovary, uterus placenta	Cyto: 179.2; Syncytio: 507.9; extravillous: 365.5; Endometrium 321.4	DDIT3, JUN, JUNB, MDM2, TP53	Regulates metabolism, immunity, inflammation, cell proliferation, and apoptosis
RELA	Ovary, uterus placenta	Cyto: 23.0; Syncytio: 47.7; extravillous: 27.7; Endometrium 24.8	BRD4, CREBBPEP300, NFKB1, NFKB1A	Regulate genes involved in apoptosis, inflammation, the immune response, and proliferation
YY1	Ovary, uterus placenta	Cyto: 121.3; Syncytio: 177.1; extravillous: 126.4; Endometrium 129.9	EP300, HDAC2, HDAC3, MBTD1, RUVBL2,	Regulates several biological functions—embryogenesis, differentiation, replication, and cellular proliferation

Table 6. Cont.

Hub Gene	Tissue Expression	Single-Cell Normalized Expression (nTPM)	Associated Genes	Functions
EP300	Ovary, uterus placenta	Cyto: 17.7; Syncytio: 34.4; extravillous: 19.0; Endometrium 49.1	CITED2, HIF1A, SMAD3, TCF3, TP53	Regulates cell growth and division and prompts cell maturation and cells to take specialized functions
CREB1	Ovary, uterus placenta	Cyto: 30.1; Syncytio: 18.7; extravillous: 25.9; Endometrium 37.8	CREBBP, CRTC2, EP300, RPS6KA5, TP53	Regulates proliferation, migration, and invasion of cells
NR3C1	Ovary, uterus placenta	Cyto: 48.6; Syncytio: 36.6; extravillous: 44.2; Endometrium 28.5	HSP90AA1, NCOA1, NCOA2, NCOR, SMARCA4	Regulates hypothalamic–pituitary–adrenal (HPA) axis by modulating availability of cortisol
STAT5A	Ovary, uterus placenta	Cyto: 1.2; Syncytio: 1.3; extravillous: 2.9; Endometrium 5.0	EGFR, ERBB4, JAK1, JAK2, JAK3	Relates IL2 signaling, modulates cytokine and growth factor action, modifies chromatin organization
STAT1	Ovary, uterus placenta	Cyto: 13.7; Syncytio: 7.9; extravillous: 60.8; Endometrium 45.2	CREBBP, JAK2, PIAS1, STAT2, STAT3	Regulates proinflammation and immune function

Cyto—Cytotrophoblast; Syncytio—syncytiotrophoblast; extravillous—extravillous trophoblast; Endometrium—endometrial stromal cells.

4. Discussion

Recent advances in high-throughput techniques transform experimental data into biological connotations. In illustrated networks, the nodes representing proteins, transcripts, or metabolites are linked by edges to show the interactions among nodes. Protein network exploration depicts the role of an individual protein and its communication with other proteins, representing the protein–protein interaction.

Centrality (network-based ranking of biological components) has been largely used to find important nodes in larger networks [17,18]. These nodes with higher degrees are more likely to be essential proteins influencing biological processes. These molecular markers and their properties are helpful when prioritizing them for disease associations. Using these methods, key biological mechanisms involved in the pathogenesis of PE were identified in the current study.

In this study, the gene–miRNA interaction networks of differentially expressed genes between PE and normal placentae revealed interactions with up to 28,000 genes and miRNAs. This shows the importance and depth of their involvement in the regulatory and interactive functions. Betweenness centrality measures the extent to which a miRNA/gene lies on paths between other miRNAs/genes. MicroRNAs/genes with high betweenness may have substantial influence within a regulatory network by virtue of their control over passing information between others [19]. It should be noted that genes with a high degree centrality are of important for the diagnosis of disease, and the proteins with a high degree of betweenness are important for drug discovery [20].

In this study, significantly upregulated (TGFB1, DUSP4, TMCC1, EMP1, and BHLHE40) and downregulated (KPNA6, ATP6V0E1, KLF6, PLEKHG2, SIKE1, and ZNF85) genes with high degree and betweenness centrality showed key roles associated to the development of PE, including cell metabolic, developmental, proliferative, differentiative and apoptotic processes; cell macromolecule biosynthesis; DNA templated transcription; and responses to enzyme binding, stress, growth factor stimulation, lipid metabolism, and hypoxia.

4.1. Upregulated Genes with High Betweenness

Transforming growth factor beta 1 is a polypeptide member of the transforming growth factor beta superfamily of cytokines. It is a secreted protein that performs many cellular functions, including the control of cell growth, cell proliferation, cell differentiation, and apoptosis [21]. TGF- β 1 signaling occurs by its binding with its receptor type 2 (TGFB2), which in turn recruits and phosphorylates TGFB1, forming a heterodimeric complex [22]. Once TGFB1 is phosphorylated, it can downstream phosphorylate proteins SMAD2 and SMAD3, which then recruit SMAD4, translocate to the nucleus, and regulate the transcription of TGF β 1 target genes [23,24]. TGF β 1 levels were elevated in women with severe and mild preeclampsia late in gestation (mean gestational age, 40 weeks) compared with normotensive pregnant women [25–27]. TGF β 1 plays a decisive role in altering dNK (decidual natural killer) phenotype and function, which may have an obvious effect on the

pathogenesis of preeclampsia [20]. In the decidual zone of normal pregnancy, the dNK cell-mediated immune response and angiogenesis were subtly regulated by Treg cells via soluble TGF β 1. However, in PE decidua, excessive amounts of TGF β produced by Treg cells could significantly impair the phenotype and function of dNK subpopulations. This distorted immune response may further damage decidual angiogenesis and cause pathological pregnancy [28]. In this investigation, TGFBR1 illustrated degree and betweenness scores of 129 and 62,386.6. The higher a gene's/protein's betweenness, the more important they are for the efficient flow of gains in a network, and downregulation of TGFBR1 would have had a significant impact on the biological functions and on the pathogenesis of PE.

The Dual-specificity phosphatase (DUSP) gene family is characterized by highly conserved amino acid sequences, implicated in a variety of biological functions [29]. Taurine upregulated 1 (TUG1) was downregulated in the placental tissues of PE patients compared with a control group [30]. TUG1 affected trophoblasts' biological function, including cell growth, migration, and crosstalk *in vitro*, and promoted the progression of preeclampsia. TET3 (tet methylcytosine dioxygenase 3, a DNA-binding protein) and DUSP were negatively regulated by TUG1. Molecular and functional interaction between TET3 and DUSPs impaired spiral artery remodeling in PE [30]. Downregulated TUG1 increased the expression of DUSP4 at both mRNA and protein levels. Notably, silencing of suppressor of variegation 39 homolog 1 (SUV39H1) by siRNAs significantly upregulated DUSP4, signifying the binding of TUG1 and SUV39H1 in the nucleus [30]. TET3 activated gene transcription by promoting DNA demethylation [31]. TET3 knockdown markedly decreased the cellular expression of DUSP4. In uterine cells, TET3 deficiency increased methylation of DUSP4 promoters. Further, the methylation level of DUSP4 promoters in the preeclamptic placenta was significantly increased compared with controls [30]. Overexpression of miR-218 (upregulated in this study; degree, 5 and betweenness, 609.1) significantly upregulated FOXP1 and TUG1 and downregulated DUSP4, at both mRNA and protein levels [30]. The regulatory network mediated by TUG1 and DUSP4 seems to be an essential determinant of the pathogenesis of PE, which regulates cell growth. In mice, the DUSP9 gene located on the X chromosome performs an essential function during placental development [31]. Mouse embryo lethality between 8 and 10.5 days postcoitum was due to a failure of labyrinth development. This correlates with the normal expression pattern of DUSP9 in the trophoblast giant cells and the labyrinth of the placenta.

Furthermore, TMCC1 was significantly downregulated in PE placentae compared with normal placentae [32]. EMP1 is a protein-coding gene involved in apoptosis, which negatively regulates cell growth [33]. Circulating EMP1 was positively associated with severe placental insufficiency, placental dysfunction, and fetal growth restriction [34]. BHLHE40 is a transcriptional repressor that responds to hypoxia and negatively regulates miR-196a-5p expression. BHLHE40/miR-196a-5p is involved in PE pathogenesis [35]. Knockdown of BHLHE40 or upregulation of miR-196a-5p restored cell viability, migration, invasion, and matrix metalloprotein (MMP)-2 and MMP-9 expression under hypoxia. BHLHE40 knockdown alleviated PE symptoms in pregnant C57/BL6N mice.

4.2. Downregulated Genes with High Betweenness Centrality

Karyopherin α 6 (KPNA6, importin α 7), directly interacts with the Kelch-like ECH Associated Protein 1 (KEAP1) [36]. Overexpression of KPNA6 facilitates KEAP1 nuclear import and attenuates the Nuclear Factor Erythroid 2-related Factor 2 (NRF2/NFE2L2) signaling, whereas knockdown of KPNA6 slows down KEAP1 nuclear import and enhances the NRF2-mediated adaptive response induced by oxidative stress [37]. Thus, KPNA6-mediated KEAP1 nuclear import plays an essential role in modulating the NRF2-dependent antioxidant response and maintaining cellular redox homeostasis [38]. In preeclampsia, there was increased decidual oxidative stress, NRF2-regulated gene expression was reduced, and KEAP1 protein expression was increased in areas of high trophoblast density [39]. This signifies the role of KPNA6. The degree and betweenness centrality scores for KPNA6 were 223 and 161,133.4. Regulatory networks mediated by KPNA6, KEAP1, and NRF2 are

essential determinants of the pathogenesis of PE, which regulates oxidative stress. ATPase H⁺ Transporting V0 Subunit E1 (ATP6V0E1/ATP6H) gene-regulated macro-autophagy was implicated in the pathogenesis of PE. ATP6H knockdown resulted in antiproliferative and apoptosis effects on BxPC-3 cells (pancreatic ductal adenocarcinoma cell line).

In normal pregnancies, placental autophagy is critical for the maintenance of cellular homeostasis that is needed for embryo and placental development [40]. Autophagy is activated in response to environmental stress, and dysregulation of autophagy is associated with various diseases [41]. Oxidative stress and hypoxia in preeclampsia are associated with an increase in the autophagic process, particularly in nutrient-deprived conditions [42]. Mitochondria are involved not only in ATP production but also in calcium homeostasis, free radical generation, cell survival, apoptosis, and necrosis [43–46]. Changes in mitochondrial dynamics, and apoptosis, are observed in preeclampsia [47]. Modification in mitochondrial gene expression influences mitochondrial homeostasis, ensuing mitochondrial dysfunction. This dysfunction leads to excessive ROS and inadequate ATP production [47,48]. Mitochondrial DNA (mtDNA) is speculated to be the marker of this dysfunction because of its inflammatory response. Oxidative stress causes membrane potential changes, inducing mitochondrial membrane depolarization and increased permeability. These disruptions will release damaged mitochondrial components, such as ROS and mtDNA, in the cytosol. As a result, there will be alteration in inflammatory and apoptotic pathways [49].

In PE, the mitochondrial apoptosis process seems to be highly altered [50]. There was a decrease in proapoptotic proteins such as p53 and BCL2-associated X and an increase in antiapoptotic proteins such as B-cell lymphoma 2 (BCL2) in term preeclamptic syncytiotrophoblast mitochondria compared with the increase in the BAX/BCL2 ratio in preterm preeclampsia [39]. In addition, soluble fms-like tyrosine kinase 1 (sFlt-1), which has antiangiogenic activity, exerted roles in oxidative stress and apoptotic pathways [51–53]. Differential apoptosis signaling in preterm and term placentae suggests that mitochondria promote cell survival in the placenta by suppressing the apoptosis mechanism. The regulation of programmed cell death and adequate antioxidant activity is important to improve mitochondrial adaptation and function [54]. Mitochondrial dysfunction due to excessive ROS production and reduced antioxidant capacity may result in an exaggerated apoptotic rate, placentation defect, and, therefore, preeclampsia.

The transcription factor Krüppel-Like Factor 6 (KLF6) has important roles in cell differentiation, angiogenesis, apoptosis, and proliferation. Furthermore, KLF6 is required for proper placental development [55]. KLF6 is present in both the early and late onset of severe-type PE [56]. KLF6 may mediate some of the effects of hypoxia in placental development and so has relevance in the development of PE.

PLEKHG2 is involved in cellular development, cellular assembly, and organization activity in early pregnancy and PE [57]. Decreased gene and protein expression of PLEKHG2 is involved in the breakdown of extracellular matrix proteins and tissue re-modeling activity in the human placenta [58]. Differentially expressed ZNF85 is involved in the top 10 GO terms, including DNA and ion bindings, between preeclampsia cases and controls [59]. In placental tissue, there was a correlation between ZNF85 expression and CpG methylation variation [60].

4.3. Comparison of miRNAs of Different Types of Preeclampsia

For comparison of different types (early- vs. late-onset; mild vs. severe) of preeclampsia, we selected DE genes in early-onset severe preeclampsia, late-onset severe preeclampsia, and late-onset mild preeclampsia from RNA-seq on 65 high-quality placenta samples that included 33 from 30 PE patients and 32 from 30 control subjects reported by Ren et al., 2021 [16]. These DE gene sets representing different types of PE were subjected to gene-miRNA interaction analysis. The top 20 molecular markers (genes and miRNAs with high betweenness) were compared, and the common six miRNAs (hsa-mir-124-3p, hsa-mir-1-3p, hsa-mir-146a-5p, hsa-mir-16-5p, hsa-mir-27a-3p, and hsa-mir-34a-5p) signifying all three types of PE types were identified. It is interesting to note that five (hsa-mir-1-3p, hsa-mir-146a-5p, hsa-mir-16-5p, hsa-mir-27a-3p, and hsa-mir-34a-5p) of these six miRNAs were the

top miRNAs (with high betweenness) exemplified from the current analysis. Their roles governing placenta development and PE are discussed below.

The significant alterations in the expression level of miRNA and the gene pairs hsa-miR-1-3p/ANXA2 and hsa-miR-1-3p/YWHAZ were associated with extracellular matrix organization, blood vessel development, smooth muscle contraction, angiogenesis, endothelial damage, and thrombi formation that caused a pulse increase in the right uterine and the umbilical arteries, hypoxia and oxidative stress, decreased placenta mass, and poor fetal development and weight (<10 percentile) [61].

Upregulated miR-146a-5p in the preeclamptic placentae provoked impaired trophoblast cell proliferation, poor invasiveness, and migratory capacity by inhibiting Wnt2 signaling [62]. miR-16-5p was upregulated in the placental tissue of a PE rat model [63]. miR-16-5p targeted the IGF-2 gene and downregulated its expression; consequently, it increased cell autophagy and cell death in the PE placenta [64].

In contrast, the downregulation of miR-27a-3p induced the migration and invasion of trophoblast cells into the uterine endometrium. Interestingly, the expression of miR-27a-3p was negatively related to ubiquitin-specific protease 25 (USP25) in recurrent miscarriage patients [65]. USP25 can regulate the processes of invasion and migration of different types of cells. It is reasonable that miR-27a-3p-mediated downregulation of USP25 contributes to the epithelial-to-mesenchymal transition, thereby inhibiting the migration and invasion of trophoblast cells via facilitating the Wnt pathway and regulating the miR-27a-3p/ATF3 axis [65,66].

miR-34a, a downstream gene of p53, regulates the cell cycle, apoptosis, and differentiation by targeting various target genes [67]. Elevated miR-34a has been reported to aggravate DNA damage and promote cell apoptosis [68]. Placental Growth Factor (PLGF) was a target gene of miR-34a [69]. PLGF regulates vascular endothelial growth and vascular remodeling via autocrine or paracrine mechanisms. miR-34a stimulates the proliferation of vascular endothelial cells and regulates DNA repair and apoptosis of these cells via PLGF [69]. It should be noted that hsa-miR-34a-5p was upregulated in the plasma during the first trimester in pregnant women with a high risk of preterm birth compared with normal controls. miR-34 was associated with pregnancy complications, including preeclampsia and intrauterine growth restriction [70,71].

4.4. Involvement of Hub Genes in Preeclampsia Development

ARNTL, CLOCK, NR3C1, ETS1, EGR1, NFKB1, CREBBP, SMARCA4, ESR1, RELA, CREB1, VDR, TP53, EPAS1, ARNT, VHL, SP1, E2F1, TFDP1, and RB1 proteins corresponding to the upregulated hub genes are involved in cellular proliferation, growth, and differentiation, cell metabolism, inflammation, and immune modulation in ovarian, uterine, and placental tissues (Table 5). In addition, biological rhythms and preeclampsia are linked [72], and ARNTL and CLOCK hub proteins are involved in circadian pathways. Similarly, IFNG, STAT3, NFKB1, IRF1, TBX21, STAT5B, GATA3, STAT4, JUN, SP1, GATA1, EGR1, ATF3, RELA, YY1, EP300, CREB1, NR3C1, STAT5A, and STAT1 proteins corresponding to downregulated hub genes are involved in cell survival, cellular growth and development, cell homeostasis, cell metabolism, immune modulation, and inflammation in ovarian, uterine, and placental tissues (Table 6). This demonstrates that the aberration of these hub genes results in PE instead of normal pregnancy.

4.5. Hub Genes with Diagnostic and Therapeutic Perspectives

Betweenness centrality measures the extent to which a miRNA/gene lies on paths between other miRNAs/genes. MicorRNAs/genes with high betweenness may influence information passing between others within the network [73]. The top three upregulated hub genes with high degree and betweenness scores were TGFBR1, DUSP4, and TMCC1. The top three downregulated hub genes with high degree and betweenness scores were KPNA6, ATP6V0E1, and KLF6. The higher a protein's betweenness, the more important it is for the efficient flow of goods in a network. It should be noted that proteins with a high

degree centrality are of important for the diagnosis of disease, and proteins with a high degree of betweenness are important for drug discovery [74].

Differentially methylated circadian clock genes ARNTL1, CLOCK, and BHLHE40 were observed in umbilical cord leukocytes and placental tissue in PE [75]. ARNTL and CLOCK are positive activators and drive the transcription of clock genes by binding to E-box elements on their promoters. The DNA methylation status of the circadian clock and clock-controlled genes in placental tissue and umbilical cord leukocytes is different between patients with EOPE and normal controls. This may be explained by a longer exposure to placental oxidative stress as compared with pregnancies complicated by late-onset preeclampsia. In term PE patients, the most enriched pathways that were correlated were hypoxia-related pathways and the membrane trafficking and autophagy-related pathways, which increased or decreased, respectively. Furthermore, CLOCK mRNA and protein expressions were reduced in the term PE placenta [76]. This suggests that circadian clock genes could be plausible candidates for the pathogenesis and etiology of PE.

The present work contains extensive bioinformatic analysis of genes, microRNAs, proteins, and biological processes between preeclampsia and normal pregnancy. However, this study may have limitations. The retrospective data extensively analyzed in this current study were originally obtained from relatively small biological samples. However, five-fold mean differences in relative expressions were used in this study. To detect these differences with adequate statistical power ($1 - \beta = 0.8$) and statistical significance ($\alpha = 0.05$), at least three samples per group were needed. The exact age (absolute age) of the pregnancy was not provided rather than stating that the normal and preeclampsia placental samples were obtained from less than 32 weeks of pregnancy. Early- and late-onset preeclampsia both result from the same problem, utero-placental malperfusion, which has different causes [77]. It has been suggested that early-onset preeclampsia is more strongly associated with internal placental factors, whereas the late-onset preeclampsia form may be primarily due to predisposing maternal factors. Some studies [78,79] found that the effect of risk factors varies according to the subtype of preeclampsia, whereas others did not [80]. Further, specific PE-related pregnancy complications are not distributed evenly across ages [81].

The association between the expression of placental tissue miRNAs and circulating miRNAs would help identify diagnostic and prognostic biomarkers. Cirkovic et al. (2021) observed increased miRNA-155 expression in both the placental tissue (SMD = 2.99, 95%CI = 0.83–5.14) and peripheral blood of women with PE (SMD = 2.06, 95%CI = 0.35–3.76) compared with women without PE [82]. However, an increased expression of miR-16a in placental tissue and significantly lower expression in peripheral blood of women with PE (SMD = -0.47, 95%CI = -0.91 to -0.03) was also observed. Several studies generated potential biomarkers utilizing samples from established PE, with less focus on prediction [83–87]. It is conceivable that coalescing biomarkers derived from different sources (multiple organ and cellular sources) may yield the best prediction. Utilizing large prospective cohort collections in unselected populations provides the best avenue for discovering novel biomarkers. However, miRNA expression differs according to the severity of PE [88] and during normal pregnancy [89]. So, these markers or combinations must be rigorously validated in external cohorts to ensure they achieve their potential to improve outcomes for pregnant people and their babies.

5. Conclusions

The evidence summarized in this article reveals the role of miRNAs in the pathogenesis of PE. The pathogenesis of PE is apparently determined by a range of miRNA molecules and their target genes and the degree of changes in their expression levels, which are associated with impairment of vascular and cellular development, circadian dysregulation, inflammation, and immunosuppression at the fetal–maternal interface, ultimately leading to impaired placental growth and hypoxic injury, which generally manifest as placental insufficiency. These miRNAs, genes, or proteins differentially expressed in placental tissue and in circulation can serve as novel diagnostic and therapeutic targets.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cimb46040216/s1>. Supplementary File S1: Transcriptional profiling of mRNA (up- and downregulated at 5-fold relative expression) between PE and normal placentae. Supplementary File S2: STRING-based Gene Ontology terms for upregulated genes. Supplementary File S3: STRING-based Gene Ontology terms for downregulated genes. Supplementary File S4: Enrichment path from ClueGo nested network analysis.

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