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Bioinformatics approach reveals the critical role of TGF-β signaling pathway in pre-eclampsia development

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ABSTRACT

Background: Pre-eclampsia is a common pregnancy complication, affecting 5–8% of pregnancies worldwide. The specific mechanism of pre-eclampsia remains unclear.
Objective: In this study, we aimed to apply bioinformatics approach to reveal related pathways or genes involving in the development of pre-eclampsia.
Study design: The gene expression profiles of GSE9984 and GSE4707 were downloaded from the Gene Expression Omnibus database. Differentially expressed genes analysis was performed by GEO2R. The Database for Annotation, Visualization and Integrated Discovery (DAVID) was applied to analyze the functional enrichment, gene ontology and Kyoto Encyclopedia of Genes and Genomes pathway of the differentially expressed genes. Gene Set Enrichment Analysis (GSEA) was conducted using the software GSEA v3.0. Protein-protein interaction (PPI) relationships were evaluated by the Search Tool for the Retrieval of Interacting Genes (STRING) and network visualization was constructed by the Cytoscape. Cell count kits-8 (CCK-8), transwell migration assay and tube formation assay were performed.
Results: A total of 160 common differentially expressed genes were extracted. Transforming growth factor (TGF) beta signaling pathway was shown to be notable in the development of pre-eclampsia. ENG, a key gene of TGF-β signaling pathway, inhibited the proliferation, migration and invasion of both HTR-8/SVneo cells and human umbilical vein endothelial cells (HUVECs), and additionally suppressed the capillary formation of HUVECs.
Conclusion: Bioinformatics approach combined with cell experiments in this study revealed that TGF-β signaling pathway was critical for the development of pre-eclampsia, and efficient biomarkers underlying this pathway need to be further investigated.

Introduction

Pre-eclampsia is a common pregnancy complication that occurs after 20 weeks of gestation. It has been reported to affect 5–8% of pregnancies worldwide [1]. The clinical characteristics of pre-eclampsia are hypertension, proteinuria, headaches, swelling and blurred vision accompanied by risk of fetal complications including intrauterine growth restriction and stillbirth. Based on the American Congress of Obstetricians and Gynecologists Committee on Practice Bulletin-Obstetrics, pre-eclampsia is clinically classified into mild and severe forms according to the hypertension and proteinuria level [2]. Pre-existing conditions of the mother including maternal age, parity, diabetes mellitus, body mass index, renal disease and hypertension may contribute to the development of pre-eclampsia [3]. Delivery of the fetus and placenta is known to be primary treatment for pre-eclampsia, as the placenta has been shown to be the origination of various pathological processes of pre-eclampsia development. However, the underlying mechanism of pre-eclampsia development still remain obscure. Reliable means for diagnosis and prediction of pre-eclampsia are still lacking, nor effective therapy or drugs to treat the disease.

Vasculogenesis and extensive angiogenesis are indispensable for placental development, and it's well known that dysfunctions in these processes will lead to adverse pregnancy outcomes. In normal pregnancy, the uterine spiral arteries with their coated muscular and elastica were deeply invaded by the extravillous trophoblasts prior to the replacement of the vascular endothelial cells [4]. Abnormal cytotrophoblast invasion to maternal spiral

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arteries leads to dysfunctional remodeling of uterine spiral arteries and placental hypoxia, which induces generalized vascular endothelial activations that are identified as pre-eclampsia syndrome [5]. Cytotrophoblast invasion to uterine spiral arteries and embryo implantation are a series of inflammatory events, which are indispensable for successful reproduction [6]. Normally, innate immune responses are enhanced and adaptive immune responses are modulated to maintain the immune tolerance to the fetal allograft in normal pregnant women. Nevertheless, in pre-eclampsia pregnant women, innate immune responses are over activated, accompanied with the dysregulation of adaptive immune responses [7]. It has been reported that normotensive pregnancy is of mild/low-grade inflammation when compared to the non-pregnant and systemic activations of maternal inflammatory cell and pro-inflammatory cytokine responses occur in preeclampsia [8]. That is, angiogenic factors from inflammatory cells directly or indirectly promote or inhibit vasculogenesis and angiogenesis at maternal–fetal interface, which may consequently induce pre-eclampsia [9]. Pre-eclampsia development also involves in other multi-factors of the dysfunctional placentation, including vascular homeostasis disruption in the placenta [10], placental transcriptome and DNA methylation modification [11,12], reduced 2-methoxyestradiol level [13] and autophagy [14] etc. Although the regulation of these processes have been extensively investigated, the main cause of pre-eclampsia still remains unclear.

In this study, we applied bioinformatics approach to reveal the systematic pathways involving in the development of pre-eclampsia. The gene expression profiling analysis, which has been widely used, is effective to distinguish the differentially expressed genes. To improve our understanding of the pathogenesis of pre-eclampsia, we searched for the gene expression profiles of normal human placenta of different gestational age and pre-eclampsia human placenta microarray data in the Gene Expression Omnibus (GEO) database. As some significant biological processes were considered to be common either in normal human placentation from the first trimester to the term or the development of pre-eclampsia from the early stage of pre-eclampsia to the late stage, such as the vasculogenesis and angiogenesis and immune responses, we also selected the datasets from normal human placenta of different gestational age. By the analysis of their biological functions, this present study aimed to identify critical genes or pathways contributing to pre-eclampsia and explored the underlying biomarkers for diagnosis, prognosis and therapy of pre-eclampsia.

Materials and methods

Bioinformatics approach

Microarray data

The gene expression profiles of GSE9984 and GSE4707 were downloaded from GEO database. GSE9984 profiled gene expression in human placenta of different gestational age based on Affymetrix GPL570 platform (Affymetrix Human Genome U133 Plus 2.0 Array) [15]. GSE4707 profiled gene expression in normal and pre-eclampsia human placenta based on Agilent GPL1708 platform (Agilent-012391 Whole Human Genome Oligo Microarray G4112A) [16]. The GSE9984 and GSE4707 contained 12 samples and 14 samples, respectively.

Identification of differentially expressed genes

Differentially expressed genes analysis for the two datasets was carried out using GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/), which performed comparisons on original submitter-supplied processed data tables using the GEO query and limma R packages from the Bioconductor project. The *P*-value of the differentially expressed genes were identified by using a classical student's *t*-test. As the distribution of values across samples are not the same in the two datasets (Table 1), adjusted *P*-value < 0.01 in GSE9984 and *P*-value < 0.05 in GSE4707 with a change $\geq$ fourfold were set to preserve the statistically significant differentially expressed genes. The common differentially expressed genes of the two datasets was selected by Draw Venn Diagram web tool (http://bioinformatics.psb.ugent.be/webtools/Venn/. Hierarchical clustering analysis was applied to categorize the data. Heatmap was drawn by HemI 1.0 software [17].

Gene ontology (GO) and pathway enrichment of differentially expressed genes

Gene ontology (GO) analysis is commonly used for gene annotation including molecular function (MF), biological processes (BP) and cellular components (CC) [18]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/) is a knowledge database for assignment of sets of differentially expressed genes to specific pathways, linking genomic information with higher order functional information [19]. DAVID (http://david.ncifcrf.gov/) was applied to analyze the functional enrichment, GO enrichment and KEGG pathway of the differentially expressed genes, and visualize the genes in the relevant biological annotation [20]. Gene Set Enrichment Analysis (GSEA) was conducted using the software GSEA v3.0 (www.broadinstitute.org/gsea/) [21,22]. Genevestigator software (https://genevestigator.com/gv/) was applied to visualize the differentially expressed genes involving in MF, BP and CC. *P* value less than 0.05 was considered statistically significant.

Construction of a protein-protein interaction (PPI) network

To evaluate the PPI network, the differentially expressed genes were mapped using STRING, the Search Tool for the Retrieval of Interacting Genes (STRING: version 10.5, https://string-db.org/) online database for evaluation of the protein–protein interaction (PPI) information [23]. Experimentally validated interactions were included and single nodes without interactions were excluded. PPI networks visualization was operated by the Cytoscape software (version: 3.5.1) [24].

Validation of key genes

BioGPS for genes search

BioGPS, a free extensible and customizable gene annotation portal and a complete resource for learning about gene and protein function, was applied to search for the gene expression of key genes in different organs according to the literatures [25].

Table 1
Small interfering RNA sequences (siRNA) and primers.

Name	Sense or Forward sequences	Anti-sense or reverse sequences
ENG siRNA	5'-GCAAUGAGGCGGUGGUCAATT-3'	5'-UUGACCACCGCCUCAUUGCTT-3'
ENG siRNA scramble	5'-GCAGGAUAGGAGGUUCCGATT-3'	5'-UCGGAACCUCCUAUCCUGCTT-3'
ENG primers	5′-ATAGGACTGTCTTCATGCGC-3′	5′-GTAGATGTACCAGAGTGCAGC-3′
GAPDH primers	5'-TTGATGGCAACAATCTCCAC-3′	5′-CGTCCCGTAGACAAAATGGT-3′

ENG: endoglin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Cell culture and treatments

The HTR-8/SVneo cells and the human umbilical vein endothelial cells (HUVECs) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 (11875-093; Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), at 37℃ incubator with 5% CO_2. Overexpression of endoglin (ENG) was achieved by a pLKO lentiviral vector targeting ENG. Lentivirus stocks were constructed using the ViraPower™ Lentiviral Packing Mix and the 293FT cell line according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc., Shanghai, China). Knockdown of ENG in HTR-8/SVneo cells and HUVECs was achieved by siRNA. Cells were transfected with ENG-siRNA at a final concentration of 50 nM using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells with scramble sequences were set to be the control group. ENG primers and ENG-siRNAs sequences were shown in Table 1.

Quantitative real-time polymerase chain reaction

Total RNA extraction, cDNA conversion and RT-qPCR assays have been described previously [26]. Each well contains 1 μl cDNA template, 0.2 μl each primer, 3.6 μl diethyl pyrocarbonate-H_2O and 5 μl SYBR Green dye. The amplification cycling conditions were list as follows: Pre-denaturation at 95 °C for 30 s; denaturation at 95 °C for 5 s, annealing at 60 °C for 10 s and extension at 72 °C for 30 s for a total of 40 cycles. Relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method and GAPDH was normalized as an internal control.

Cell proliferation and transwell and tube formation assays

For the cell proliferation assay, post-transfected cells were suspended in RPMI 1640 medium for 3 days in 96-well plates (5×10^4 cells/well). CCK-8 was subsequently mixed and cells were incubated at 37 °C for another 2 h. The absorbance was measured using an ELISA reader (Tecan Group, Ltd., Mannedorf, Switzerland) at a wavelength of 450 nm. For the cell migration and invasion assays, transwell assay were performed, post-transfected cells were cultured in 200 μl medium before transferred onto the upper

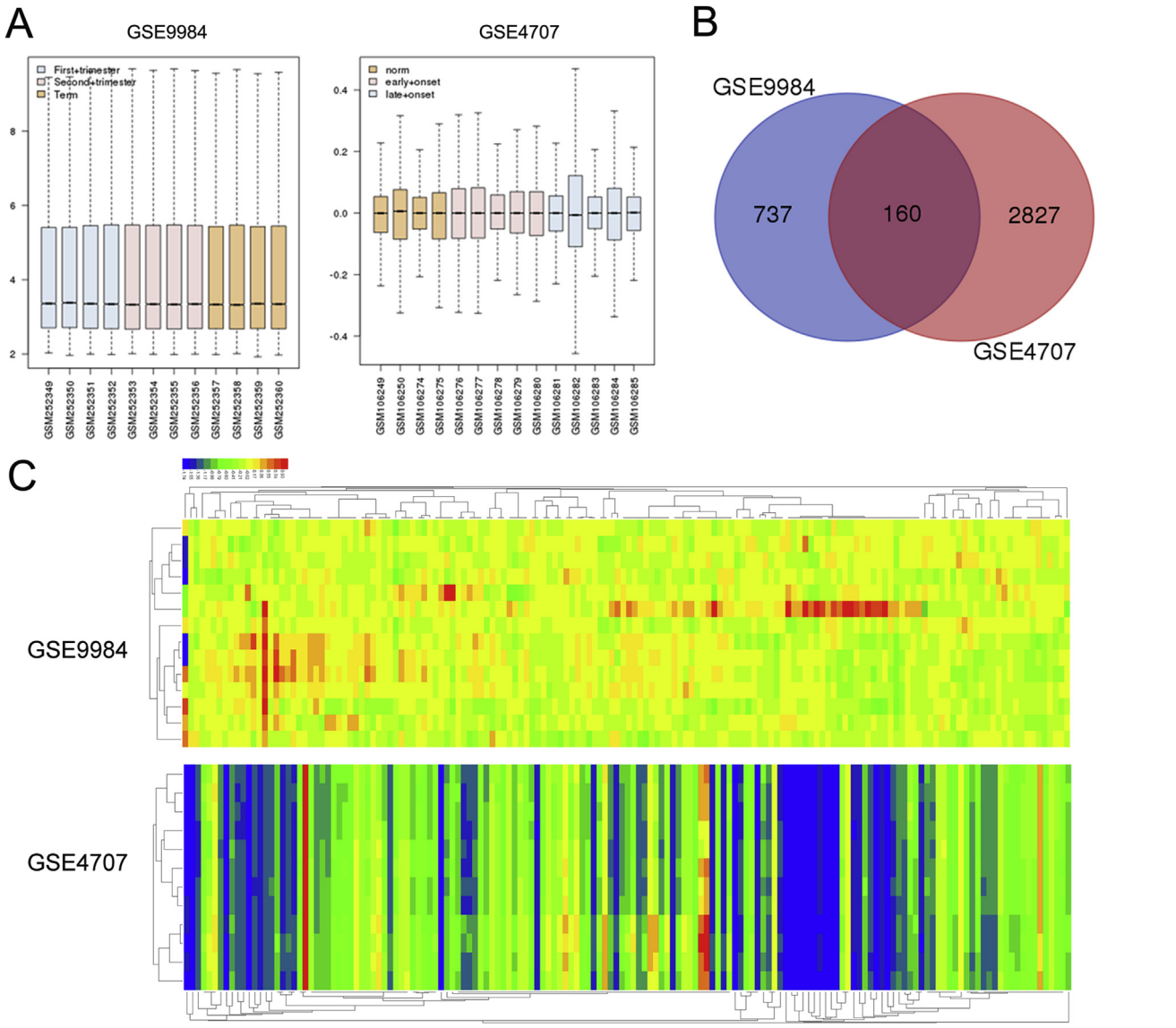

Fig. 1. Overview of GSE9984 and GSE4707 and identification of common differentially expression genes. A: The box plot graph of value distribution of GSE9984 and GSE4707. B: Cutoff values and venn diagram displaying the number of differentially expressed genes. C: Hierarchical clustering and heat maps of the common differentially expressed genes in GSE9984 and GSE4707.

chambers of 24-well plates (5×10^5 cells/well) with or without a Matrigel coated. The lower chamber was added with 800 μl medium of 10% FBS. After 24 h of incubation, cells in the lower chamber were fixed with absolute methanol and stained with 0.1% crystal violet solution (Sigma Aldrich; Merck KgaA, Darmstadt, Germany). For the tube formation assay, Matrigel basement membrane matrix (BD Biosciences, Franklin Lakes, NJ, USA) was coated into a 96-well plates prior to HUVECs (1×10^5cells/well) incubation. After 6 h, capillary-like HUVEC structures were photographed. Cell counting and structure observation were operated at medium (×10) magnification using a microscope from 3 random fields. Photo analysis was performed with the software Image J software version 1.49 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Statistically analysis was performed using the Stata 13.0 software (Stata Corp LP, College Station, TX, USA). The Student's *t*-test and one way analysis of variance were applied. Data were presented as the mean ± SEM. *P* value less than 0.05 was considered statistically significant.

Results

Overview of the GEO microarray data and identification of differentially expressed genes

The box plot of datasets were provided to assess whether the distribution of values across samples were median-centered and of conformity. The results generally showed that the data were normalized and cross-comparable. However, distributional differences of samples appeared to be more obvious in GSE4707 than GSE9984 (Fig. 1A). Adjusted *P*-value < 0.01 in GSE9984 and *P*-value < 0.05 in GSE4707 with a change ≥ fourfold were set to filter the statistically significant differentially expressed genes. Total 4808 elements in GSE4707 and 1209 elements in GSE9984 were selected. Identified unique elements with Draw Venn Diagram were 2987 and 897in GSE4707 and GSE9984, respectively. Total 160 common differentially expressed genes were extracted from the two groups after the comparison (Fig. 1B). Hierarchical cluster analysis was performed on the common differentially expressed genes, and heat maps of the common differentially expressed genes of the two datasets were shown below (Fig. 1C).

Pathway enrichment analysis and key genes identification

The results of the GO analysis of the common differentially expressed genes indicated that embryonic development, transcription regulation and cell division were the main biological processes. Centrosome, microtubule and cell adherence junction were the main cellular components. The significant molecular functions were related to nucleotide binding, microtubule binding, GTP binding and GTPase activity. The results of KEGG pathway enrichment analysis suggested that biosynthesis of amino acids was the most significant (Table 2). Furthermore, the common differentially expressed genes were assigned to Genevestigator to count the number of genes via GO analysis, which was shown below (Fig. 2A). Then the PPI among the common differentially expressed genes were evaluated by STRING, and PPI networkvisualization was constructured by the Cytoscape. ENG, platelet-derived growth factor subunit B (PDGFB), stearoyl-CoA desaturase (SCD), phosphoglycerate kinase 1 (PGK1) and inhibin beta A subunit (INHBA) ect. were preserved according to the inclusion criterion (Fig. 2B). To further identify the key genes, GSEA was conducted on GSE9984 and GSE4707 datasets using the GSEA software (Table 3). "TGF_BETA_SIGNALING", "TNFA_SIGNALING_VIA_NFKB", "PROTEIN_SECRETION", "P53_PATHWAY", "COMPLEMENT" and "ESTROGEN_RESPONSE_EARLY" were the common biologic characteristics between GSE9984 and GSE4707. Furthermore, TNFA signaling and TGF beta signaling were verified to be of significance via comparing the selected genes with the enriched gene sets. TNFA signaling and TGF beta signaling enrichment plots were shown below. The enrichment score in both signaling pathways were positively correlated with the term of placenta, that is, the enrichment score was increased according to the increase of gestational age, while the enrichment score was lower in pre-eclampsia placentas (Fig. 2C). Finally, ENG and INHBA were considered to be the most significant key genes for their high involvement.

Table 2
GO analysis and KEGG pathway enrichment analyses of the common differentially expressed genes (P < 0.05).

Category	Term	Count	Fold Enrichment
GO-BP terms	GO:0,001,701in utero embryonic development	4	2.57
	GO:0,045,944positive regulation of transcription from RNA polymerase II promoter	11	1.34
	GO:0,006,366transcription from RNA polymerase II promoter	6	1.40
	GO:0,006,357regulation of transcription from RNA polymerase II promoter	5	1.36
	GO:0,007,067mitotic nuclear division	4	1.93
	GO:0,051,301cell division	4	1.37
	GO:0,000,398mRNA splicing, via spliceosome	3	1.62
	GO:0,007,264small GTPase mediated signal transduction	4	1.95
	GO:0,098,609cell-cell adhesion	4	1.77
GO-CC terms	GO:0,005,813centrosome	7	2.05
	GO:0,005,874microtubule	4	1.61
	GO:0,005,813centrosome	7	2.05
	GO:0,005,913cell-cell adherens junction	4	1.55
GO-MF terms	GO:0,001,228transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding	5	6.30
	GO:0,043,565sequence-specific DNA binding	6	1.39
	GO:0,003,700transcription factor activity, sequence-specific DNA binding	6	0.75
	GO:0,008,017microtubule binding	4	2.30
	GO:0,003,723RNA binding	8	1.75
	GO:0,000,166nucleotide binding	5	1.72
	GO:0,044,822poly(A) RNA binding	10	1.06
	GO:0,003,676nucleic acid binding	8	0.97
	GO:0,005,525GTP binding	5	1.56
	GO:0,003,924GTPase activity	3	1.53
	GO:0,098,641cadherin binding involved in cell-cell adhesion	4	1.65
KEGG pathway	hsa01230Biosynthesis of amino acids	4	6.12
	hsa01130Biosynthesis of antibiotics	5	2.67
	hsa01200Carbon metabolism	3	3.01
	hsa01100Metabolic pathways	11	1.01
	hsa05211Renal cell carcinoma	3	5.23
	hsa05166HTLV-I infection	4	1.77
	hsa05200Pathways in cancer	5	1.44

GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; BP: biological processes; CC: cellular components; MF: molecular function.

ENG and INHBA expression and biological characteristics and PPI network regulation

ENG and INHBA were input to BioGPS online tool to search for the gene expression profile in different organs. The results showed

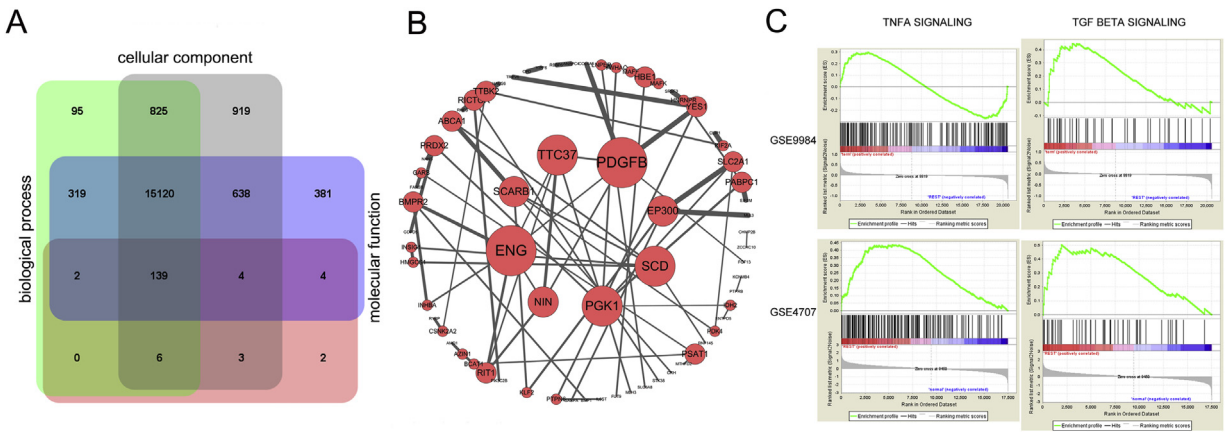

Fig. 2. Analysis of the common differentially expressed genes. A: Number of the common differentially expressed genes under GO analysis via Genevestigator software examination. B: PPI relationships and its visualization of the common differentially expressed genes via STRING and Cytoscape. C: Enrichment plot of TNFA signaling and TGF beta signaling via GSEA software. DEGs: differentially expression genes; GO: gene ontology; PPI: protein-protein interaction; STRING: Search Tool for the Retrieval of Interacting Genes; TNFA: Tumor necrosis factor alpha; TGF: Transforming growth factor; GSEA: Gene Set Enrichment Analysis.

Table 3
Gene set enrichment analysis of GSE9984 and GSE4707 datasets.

NAME	SIZE	ES
GSE9984		
HALLMARK_TGF_BETA_SIGNALING	50	0.448245
HALLMARK_PROTEIN_SECRETION	88	0.263461
HALLMARK_ESTROGEN_RESPONSE_EARLY	183	0.346421
HALLMARK_P53_PATHWAY	182	0.291359
HALLMARK_WNT_BETA_CATENIN_SIGNALING	40	0.288427
HALLMARK_INTERFERON_ALPHA_RESPONSE	88	0.448442
HALLMARK_ADIPOGENESIS	184	0.261939
HALLMARK_APICAL_JUNCTION	193	0.254992
HALLMARK_IL6_JAK_STAT3_SIGNALING	85	0.357378
HALLMARK_IL2_STAT5_SIGNALING	182	0.278968
HALLMARK_COMPLEMENT	192	0.286836
HALLMARK_INFLAMMATORY_RESPONSE	189	0.334133
HALLMARK_KRAS_SIGNALING_UP	192	0.306358
HALLMARK_INTERFERON_GAMMA_RESPONSE	187	0.32919
HALLMARK_COAGULATION	133	0.294726
HALLMARK_TNFA_SIGNALING_VIA_NFKB	185	0.297199
GSE4707		
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	91	0.6074786
HALLMARK_ANDROGEN_RESPONSE	89	0.5701024
HALLMARK_MTORC1_SIGNALING	173	0.53055346
HALLMARK_UV_RESPONSE_UP	148	0.38951832
HALLMARK_TGF_BETA_SIGNALING	51	0.5029877
HALLMARK_MYC_TARGETS_V1	167	0.567924
HALLMARK_GLYCOLYSIS	175	0.40217888
HALLMARK_HYPOXIA	180	0.41924852
HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY	44	0.4449219
HALLMARK_ESTROGEN_RESPONSE_EARLY	170	0.3557787
HALLMARK_HEME_METABOLISM	168	0.38773412
HALLMARK_OXIDATIVE_PHOSPHORYLATION	182	0.48985246
HALLMARK_G2M_CHECKPOINT	167	0.37452054
HALLMARK_P53_PATHWAY	167	0.36034542
HALLMARK_TNFA_SIGNALING_VIA_NFKB	180	0.43689865
HALLMARK_E2F_TARGETS	159	0.40630132
HALLMARK_FATTY_ACID_METABOLISM	134	0.33162612
HALLMARK_PROTEIN_SECRETION	83	0.4809154
HALLMARK_COMPLEMENT	180	0.3349625
HALLMARK_DNA_REPAIR	135	0.3907232

that ENG and INHBA were highly expressed in the placenta (Fig. 3A). The two-dimensional view analysis via DAVID showed that ENG and INHBA were both involved in the biological processes of transcription regulation (Fig. 3B). Furthermore, ENG and INHBA were input to STRING to search for the PPI with other possible proteins. The results of STRING showed that all of the proteins were involved in the TGF beta signaling pathway (Fig. 3C), indicating that TGF beta signaling pathway might play an important role in the development of pre-eclampsia.

Validation of the function of ENG in HTR-8/SVneo cells and HUVECs

To validate the critical role of ENG in the biological behaviours of placental cells, HTR-8/SVneo cells and HUVECs were applied. Overexpression and knockdown of ENG in cells were achieved by pLKO lentiviral vectors and siRNAs, respectively. RT-qPCR was applied to confirm transfection efficiency (Fig. 4A). For cell proliferation, the results of CCK-8 assays showed that overexpression of ENG obviously decreased proliferation capacity of HTR-8/SVneo cells and HUVECs, while knockdown of ENG remarkably increased this capacity (Fig. 4B). For cell migration and invasion, the transwell assays were carried out. As shown in Fig. 4C, migration and invasion of HTR-8/SVneo cells were significantly suppressed in the pLKO-ENG group, but promoted in the si-ENG group, when compared with the control group. The results of transwell assays in HUVECs were consistent with that of HTR-8/SVneo cells (Fig. 4D). Moreover, overexpression of ENG inhibited the organization of capillary-like structures in HUVECs in the tube formation assays while the tube-forming activity was stimulated with the knockdown of ENG, when compared with the control (Fig. 4D).

Comments

As a pregnancy-specific syndrome, pre-eclampsia caused a high risk of maternal and perinatal mortality and morbidity. Although the regulation of dysfunctional placentation, which was considered to be the originations for the etiology of pre-eclampsia, has been widely investigated, the exact mechanism of pre-eclampsia development still remains unclear. In the present study, bioinformatics approach was applied to reveal the possible pathways or critical genes related to the development of pre-eclampsia. As some significant biological functions were considered to be common either in normal human placentation or the development of pre-eclampsia, such as the vasculogenesis and angiogenesis, the gene expression profiles of normal human placenta of different gestational age (GSE9984) and pre-eclampsia human placenta (GSE4707) were analyzed. A total of 160 common differentially expressed genes were extracted, Gene expression profiling analysis

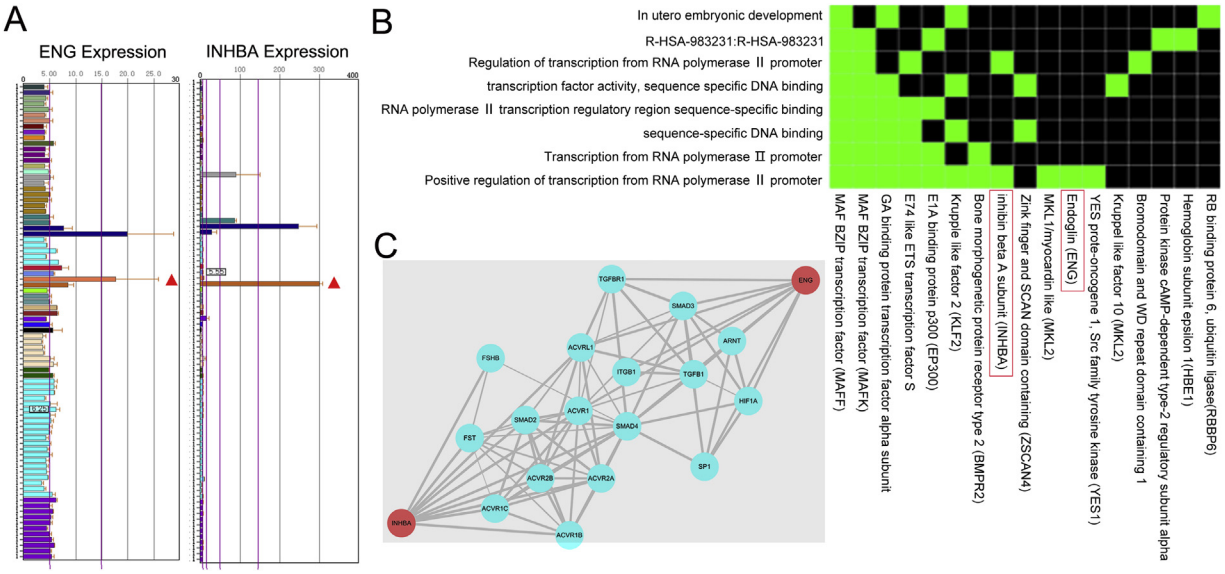

Fig. 3. ENG and INHBA expression and biological characteristics and PPI network. A: ENG and INHBA expression in different organs.Red solid triangle: ENG or INHBA expression in placenta; B: Two-dimensional view analysis of ENG and INHBA. Red hollow rectangle: ENG and INHBA; C: PPI network of ENG and INHBA with other proteins. ENG: endoglin; INHBA: inhibin beta A subunit (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

as well as PPI network showed that TGF beta signaling pathway was of the most significance. As a key gene of TGF beta signaling pathway, ENG was then selected for the following experiments of cell biology and morphology to confirm the critical role of TGF beta signaling pathway in the development of pre-eclampsia. Our results showed that ENG inhibited cell proliferation, migration and invasion of both HTR-8/SVneo cells and HUVECs, and additionally suppressed the capillary formation of HUVECs, suggesting that ENG played a critical role in the development of pre-eclampsia via the down-regulation of TGF-β signaling pathway.

The TGF-β is a member of the TGF-β superfamily which includes more than 45 members [27]. There are five isoforms of TGF-β in the TGF-β subfamily, including TGF-β1, TGF-β2, TGF-β3, TGF-β4 and TGF-β5. However, only TGF-β1, TGF-β2 and TGF-β3 are found to express in human tissues [28]. For instance, the three TGF-β isoforms are dynamically and abundantly expressed in the endometrium, and appear to involve in various biological processes, and influences various cellular functions associated with cell proliferation, differentiation and decidualization and the establishment of pregnancy via the TGF beta signaling pathway. Further, the expression of these isoforms of TGF-β have also been detected in the placenta, which undergoes rapid development and remodeling as well [29]. In normal pregnancy, the uterine wall with its underlying spiral arteries was invaded by the extravillous trophoblasts prior to the replacement of the vascular endothelial cells [4]. Extravillous trophoblasts cells differentiate from cyto-trophoblast cells, where TGF-βs and TGF-β receptors are expressed. TGF-β1 and TGF-β2 are the most abundant isoforms expressed in cytotrophoblast cells, while TGF-β3 is located in the maternal tissues. In addition, TGF-β1 is down-regulated in the invasive extravillous trophoblasts, and TGF-β2 is strongly expressed in decidual cells, while TGFb3 is presented only in immune cells such as macrophage [30,31]. It has been reported that TGF-β1 inhibits the migration and invasion of trophoblast cells partly via the up-regulations of the secretion of endogenous tissue inhibitors matrix metalloproteinases (MMP)-1 and -2 and connective tissue growth factor (CTGF) [32–34]. TGF-β3 is also considered to inhibit the trophoblast outgrowth for its inhibition results in increased trophoblast cells outgrowth, elevated MMP expression/activity [35]. Furthermore, blockade of the expression of TGF-β3 in placenta of pre-eclampsia impairs the invasive potential of trophoblast cells, suggesting that TGF-β3 may be responsible for the failed trophoblast invasion [30].

To further investigate the role of TGF-β signaling pathway in the development of pre-eclampsia, we conducted cell biological behavior experiments on transfected HTR-8/SVneo cells and HUVECs with ENG over-expression or knockdown. ENG is an auxiliary member of the TGF-specific membrane receptors, the others of which are types I (TRI) and II (TRII) [36]. And ALK5 and ALK1, the two different types of TRIs, have been reported to mediate the negative functions in endothelial cells (ECs) via ENG targeting [37]. Although ENG interacts with both ALK5 and ALK1, its interaction with ALK5 is stronger than with ALK1, suggesting that ENG balances the effects of TGF-β signal pathway on vascular pathophysiology via ALK5 or ALK1 [38]. ENG is essential for the inhibitory effects on TGF-β, and responsible for the differential actions of the TGF-β isoforms TGF-β1 and TGF-β3, but not TGF-β2 [39]. These differential actions are mediated by the interactions between ENG and the ligand binding receptor TRII [40]. It has been repeatedly confirmed that high level of the circulating soluble form of ENG is positively correlated with the occurrence of pre-eclampsia. Endoglin may affect the vascular homeostasis by recruiting the TGF-β1and preventing its binding to the membrane receptors in ECs [41].

To conclude, endoglin is a member of TGF-β membrane receptors and interacts with TGF-β1 and TGF-β3. These messages can be transferred to the downstream via TGF-β signaling pathway, which involves in the cell proliferation, migration and invasion and vessel formation ect. Our results confirmed that ENG inhibited cell proliferation, migration and invasion of both HTR-8/SVneo cells and HUVECs, and additionally suppressed the capillary formation of HUVECs. Therefore, bioinformatics approach combined with in vitro experiments in this study reveals that TGF-β signaling pathway is a critical pathway in the development of pre-eclampsia (Fig. 5). As sENG, INHBA, activins, proinflammatory cytokines in the TGF-β signaling pathway have been applied for diagnosis, prognosis of the pre-eclampsia [42,43], it is reasonable that more efficient biomarkers underlying this pathway need to be further investigated.

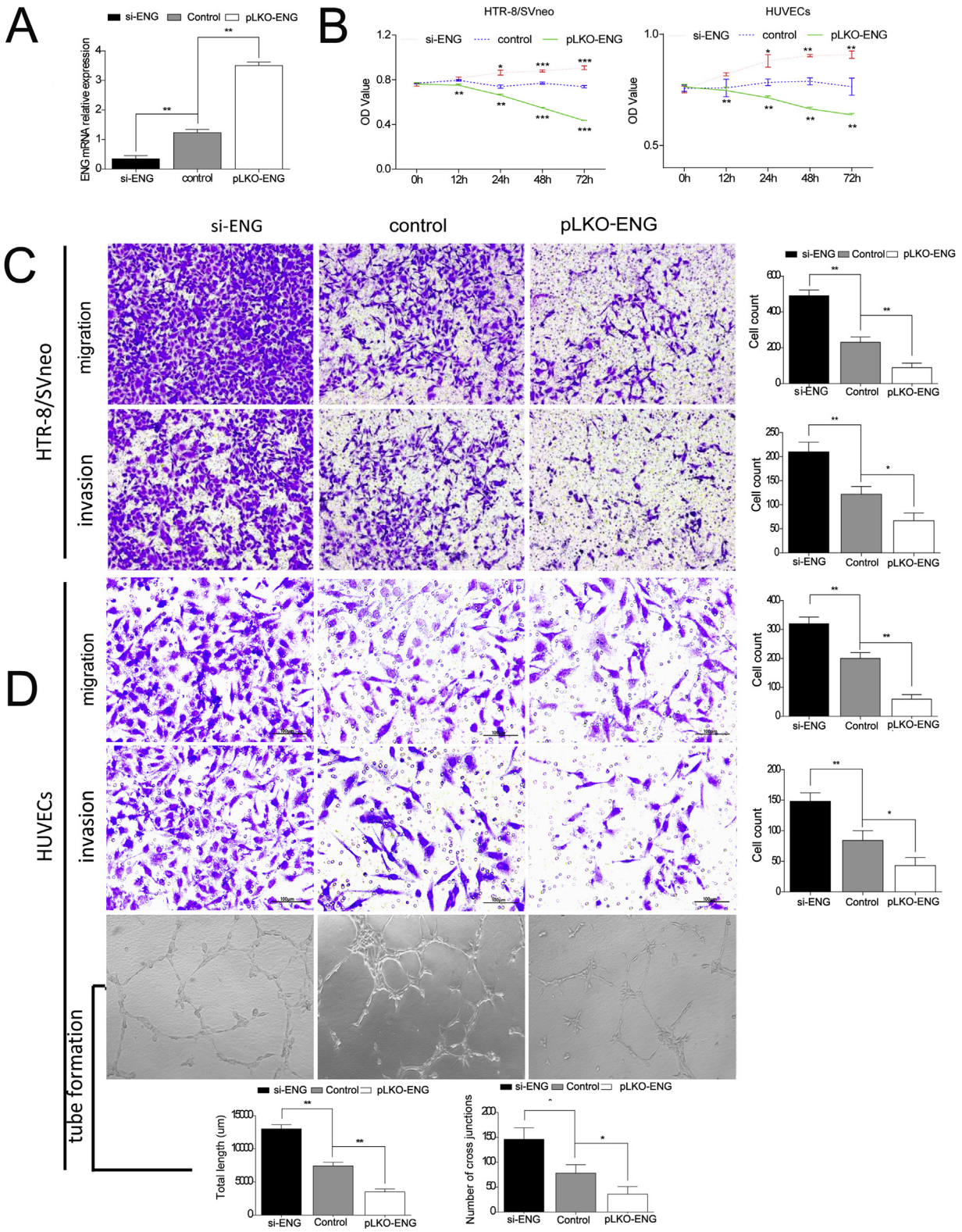

Fig. 4. ENG promotes proliferation, migration, invasion and tube formation of HTR-8/SVneo cells and HUVECs. A: Conformation of the efficacious transfection with RT-qPCR experiments. B: Post-transfected HTR-8/SVneo cells and HUVECs were cultured for 3 days and CCK-8 assays were conducted to exam the proliferation capacity. C&D: Migration and invasion capacity of transfected HTR-8/SVneo cells and HUVECs was measured by transwell migration/invasion assays after 48 h cultivation. Micrographs of capillary-like structures shaped by transfected HUVECs. Total length and number of cross junctions were measured using Image *J* software. Graphs were taken at ×10 magnification under microscope. Data are shown as the mean ± SEM from 3 independent wells. $^{*}P < 0.05$, $^{**}P < 0.01$, versus control. ENG: endoglin; HUVECs, human umbilical vein endothelial cells; CCK-8: Cell Counting Kit-8; SEM, standard error of the mean.

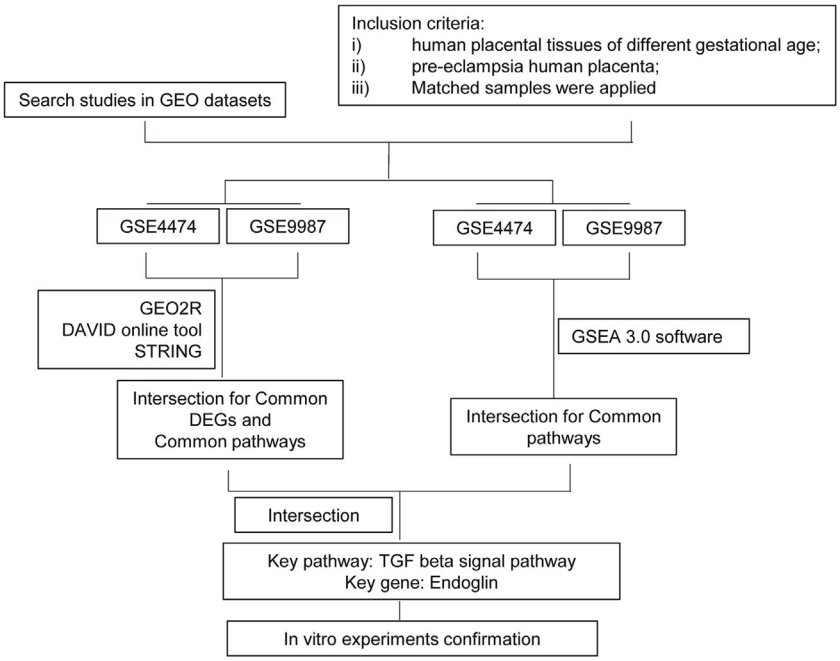

Fig. 5. Summary procedure. Flowchart showing the identification and selection of microarray studies from Gene Expression Omnibus (GEO) and the data analysis procedure and related tools.

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