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Epigenetic impact of long non-coding RNA lnc-ADAM9 on extracellular matrix pathway in preterm syndrome through down-regulation of mRNA-ADAM9

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Abstract

Aim: Evidence suggests that the risk of spontaneous preterm birth (sPTB) is the result of environmental exposure interacting with genetic risk and is mediated by epigenetic modification. Long non-coding RNA (lncRNA) comprises a large group of regulators of epigenetic modification that has recently become the focus of increased investigation in reproductive science. Human placenta expresses many lncRNAs, and differential expression profiles (DEPs) have identified several lncRNAs as associated with sPTB. However, little is known about lncRNA's role in the epigenetic modification of the genes potentially involved in sPTB. This study is to better understand the epigenetic regulation of lncRNA on the development of sPTB.

Methods: A transcriptomic analysis of human placentas derived from various pregnancy outcomes was performed as a discovery study. This was followed by a quantitative confirmation to validate the differential transcription of lncADAM9, the lncRNA overlapping with the ADAM9 gene locus, and of lncRNA-overlapped mRNA of ADAM9 (mRNA-ADAM9). *In vitro* examination of lncADAM9 transgenic (TG) HTR8 cells were used to perform functional assessment to address the role of lncADAM9-mediated epigenetic regulation of extracellular matrix-adhesion (ECM-A) associated molecules. This assessment was then expanded to studies of human fetal membranes.

Results: We observed that expression of lncADAM9 was increased in sPTB, and this increase was further associated with the down-regulation of mRNA-ADAM9 in human placentas. *In vitro*, overexpression of lncADAM9 in lncADAM9-transgenic HRT8 cells led to DEPs relevant to ECM-A molecules, particularly at the loci of CNTN1, NRXN2, SPN, ICAM2, and HLA-DPB1. This was also true in fetal membranes from abnormal versus normal fetal membranes.

Conclusion: We have studied the epigenetic impact of differentially expressed lncADAM9 on the ECM-A pathway that is associated with sPTB and documented that this impact may be mediated through the down-regulation of mRNA-ADAM9. Our results of demonstrating the epigenetic regulation of lncADAM9 on the ECM-A pathway may help provide greater insight into critical pathogenic mechanisms underlying sPTB.

Keywords: Spontaneous preterm birth (sPTB), long non-coding RNA (lncRNA), epigenetic regulation, ADAM9, extracellular matrix (ECM)

INTRODUCTION

Spontaneous preterm birth (sPTB), parturition before 37 gestational weeks (GW), is a leading cause of neonatal morbidity and mortality worldwide. Clinically, sPTB may present as spontaneous preterm labor (sPTL), defined as premature parturition with no prior rupture of fetal membrane, or as preterm premature rupture of fetal membrane (pPROM) that presents as rupture of membrane before uterine contraction. sPTB is a highly heterogeneous syndrome[1,2], resulting from gene-environment interaction that consequently causes biological changes of (epi)genetic variation and functions as a predisposing factor[3]. Many studies of gene-environment interaction in human sPTB have focused on the determination of a correlation between a monogenic factor, such as a specific genetic locus that is derived from single-omic data generated from genome-wide variation in blood or placenta with the outcome of pregnancy while associated with a specific environmental exposure[1,4-8].

Long non-coding RNA (lncRNA) is a group of transcripts that are more than 200 nucleotides without coding potential, but act as epigenetic expression regulators through several molecular mechanisms. LncRNAs have been determined to play a role in human reproduction and development[9]. It has been reported that lncRNA is a critical player in regulating epigenetic modification and is thus an important mediator of gene-environment interaction relevant to placental development including trophoblast differentiation[10,11]. In addition to miscarriage (MC), intrauterine grown restriction (IUGR), preeclampsia (PE), and gestational diabetes mellitus (GDM)[12,13], a pathogenic role of lncRNA is linked to human reproductive disorders, including sPTB[2,3]. LncRNA was found to have an epigenetic regulatory function in the relationship between social and environmental exposures and sPTB[14-18].

Studies on the pathogenic mechanisms underlying sPTB have applied an *in vitro* cell culture model to probe the interaction between genes and the intrauterine environment[19,20]. Currently, a collective, quantitative framework is lacking to relate the complex, nonlinear mechanical behavior of the placenta to changes in the pathogenic pathway, which could contribute to the clinical complex but overlap as a causal factor leading to

sPTB. Despite efforts to understand the genetic and/or genomic mechanisms associated with sPTB, little is known about the epigenetic regulation of lncRNA involved in sPTB. The insufficiency of a lncRNA transgenic model limits the ability to address the gene-environmental interactions that may result in the highly heterogenous sPTB.

Evidence obtained in our earlier studies indicates that lncRNA may be involved in regulating transcription to produce lncRNA-overlapped mRNA and gene-specific mRNA[3,21,22]. In particular, co-differentially expressed pairs of lncRNAs and mRNAs sharing the same genomic loci in sPTB were identified in the ubiquitin-proteasome system (UPS) and found to be related to the ubiquitin-proteasome-collagen (CUP) pathway[23]. Similarly, a relationship between lncADAM9 and lncADAM9 overlapping mRNA-ADAM9 was identified in the fetal membrane that is associated with sPTB[3,20]. ADAM9, a disintegrin and a metalloproteinase domain (ADAM) 9, is known to be expressed by monocytes and macrophages, and functions potently as matrix metalloproteinase-9 to degrade several proteins, including fibronectin, entactin, laminin, and insoluble elastin, which are the molecules involved in the pathways of extracellular matrix and/or cellular adhesion (ECM-A)[24]. We therefore hypothesize that differentially expressed lncADAM9 in human placentas may have a pathogenic impact on the ECM-A pathway. This impact is likely through its epigenetic regulation on the overlapped mRNA of ADAM9.

MATERIALS AND METHODS

Ethics statement

The parent study design was reviewed and approved by the Ethics Committee of Inner Mongolia Maternal and Child Health Care Hospital. Written informed consent was obtained from the pregnant women who participated in this study. All material and data were previously coded and are anonymous to the authors of this study.

Study design

The parent study was designed as two phases: discovery and confirmation, and its goal was to identify lncRNA and functionally characterize the potential epigenetic regulatory role. In the discovery study[3], lncADAM9 and the transcript at the locus where the lncADAM9 overlapped, mRNA-ADAM9, were identified by microarray analysis and were further studied to determine their differential expression profiles (DEPs). In the confirmation phase, the identified lncRNA was designed to be subjected to an *in vitro* study using transgenic cell culture in human HTR8 cells. Next, variant pathways were determined through RNA sequencing (RNA-seq). The genetic locus of ADAM9 was selected as our target in the current confirmatory functional analysis because of that ADAM9 is a membrane-anchored protein that participates in a variety of physiological functions, primarily through the disintegrin domain for adhesion and the metalloprotease domain for ectodomain shedding of a wide variety of cell surface proteins. ADAM9 influences the developmental process and inflammation[25]. Given that cellular adhesion and the metalloprotease have been determined to be associated with sPTB in our previous studies[21-23], we believe that focusing lncADAM9 regulation on the ECM-A pathway would be a logical reason to study the epigenetic impact with *in vitro* system.

Specimens

On entry into the study, previously banked specimens were selected according to their clinical outcomes of birth and GWs at parturition. These groups were defined as A, pPROM ≤ 35 GWs; B, FTB (full-term birth) at 39-40 GWs without membrane rupture; C, sPTL at ≤ 35 GWs without membrane rupture; and D, premature rupture of membranes (PROM) at 39-40 GWs. In the discovery study, 40 cases of human placentas (10 from each group) were subjected to an array-based assessment, as we have reported[3]. Additionally, 120 fetal membrane tissue samples (30 in each group) from age-matched (25-35 years of age)

were collected and divided into the four groups (30 fetal membrane samples per group) of pregnancies, as described above.

Discovery study with microarray

The discovery study was performed as we have reported elsewhere[3]. Differentially expressed lncRNAs and lncRNA-overlapped mRNAs and their involvement in pathogenic pathways were identified in human placentas. These pathways, which were constructed by the highly bioinformatic-enriched scores of DEPs of lncRNAs, included the ECM-A pathway[3,21,22].

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from human tissues of the placenta, fetal membrane, or cultured HTR8 cells by using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed using a Thermo reverse transcription kit (Thermo Fisher, Waltham, MA, USA), and qRT-PCR using SYBR Green Master Mix (Thermo Fisher). The primers used are listed in Table 1. Among them, three lncRNAs (lncADAM9, LncPCDH10, lncUSP46) and 19 mRNAs, which are involved in the ECM-A pathway[25-27], were selected as the targets for quantitative analysis. RNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control, and the expression values of lncRNAs and mRNAs were normalized. Comparisons were performed inter-group either individually (such as A *vs.* B) or combined (A + C *vs.* B + D). The data were subjected to one-way analysis of variance (one-way ANOVA) followed by an unpaired, two-tailed *t*-test. Differences were considered statistically significant at $P < 0.05$.

Overexpression of incRNAs in HTR8 cells mediated by lentivirus

PSE3569 was the lncADAM9-overexpressing plasmid constructed with an RNA-expressing lentiviral vector PMT (BoSe Biotech, Shanghai, China) inserted with a cDNA that had been generated from reverse-transcription from lncADAM9 sequence. Similarly, PSE3570, used as an experimental control, was constructed with a cDNA of lncPCDH10 inserted into PMT. The lentiviral vector PMT without insertion of lncRNA was used as a control of "empty skeleton plasmid" in transfection. Packaging and transfection of lentiviral plasmids followed a standard procedure[28]. The lentivirus packaging cell line 293T was cultured in DMEM medium with 10% FBS and antibiotics for 72 h. Lentiviral constructs containing cDNA sequence of lncADAM9, confirmed in the orientation of 5′ → 3′ transcription *via* PCR-sequencing, was packed with 293T cells and transfected into HTR8 (ATCC® CRL-3271™) that we have reported in studying IUGR[29]. The HTR8 cells were cultured in RPMI medium supplemented with 10% FBS and antibiotics. After transfection, the culture of lncADAM9-TGHTR8 cells was replaced with a fresh medium for another 72 h at 37 °C with 5% CO_2[29]. The overexpressed lncADAM9 and mRNA-ADAM9 levels were tested by qRT-PCR. Lentiviral plasmids without insertion were used as controls in transfection studies.

Transcriptomic profile

The differentially expressed genes between lncRNA-overexpressed samples and their blank control were identified as we have reported earlier[3], with the filter criteria $|\log2FC| \geq 2$ and P value < 0. 05. The target gene set was clustered with KEGG and GO functional enrichment. Fisher's exact test was carried out to calculate whether the GO/KEGG pathway function set was significantly enriched in the list of target genes, and the acquired P values were corrected to obtain FDR (false discovery rate).

Quantitative analysis of ECM-A proteins

The fetal membrane tissues were washed twice with ice-cold distilled PBS and lysed in RIPA protein extraction buffer (Sigma-Aldrich, Allentown, PA) containing protease inhibitors. Protein concentration was determined by the BCA protein determination method. The lysates (20 ng protein) were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Burlington, MA). The primary

Table 1. Primer sequences used for qRT-PCR

	Genes	Forward primers (5′-3′)	Reverse sequence (5′-3′)
lncRNA	LncADAM9	TACATACAATGGTCAGATGGCAAA	GCCTTGATGGGAACTGCTGA
	LncPCDH10	TGAGGGAACAGGTTCTCTATGGT	TGTGTGGTTCTGATGTGTCTCCTAC
	lncUSP46	CGGGGACGATCAGTCACATAAG	CGTGCTGGGATTGGTCATAGTT
mRNA	ADAM9	GCCACTGGGAATGCTTTGT	GCAGTATTCATTTCATTGTATGTAGGT
	PCDH10	TGTCCAACGAGACTAAACACCAG	CTATCTCCCTGTTCACTGTCTCCAT
	USP46	AGAAGCCCAGAAAAGGATGAGG	GAACGACCACCGCAACCAA
	CADM1	CCACAGGAAAGTTACACCACCAT	CACCTCCGATTTGCCTTTTAG
	CD274	CAATGTGACCAGCACACTGAGA	CAGTGCTACACCAAGGCATAATAAG
	CLDN7	AGGGGCTGTGGATGGACTG	GCCTTCTTCACTTTGTCGTCTC
	CNTN1	ATAGAAGTCCCAATCCCCAGAGA	GGGTGCACCTGAAATTTTGACT
	HLA-DPB1	CGGAGTAAGACATTGACGGG	CTCGTTGAACTTTCTTGCTCCT
	ICAM2	TCCAGGATCGGATGAGAAGGTA	GGAGATGTTTGAGACCAAGTAATG
	ITGAL	AGCAGCAAGCATTTCCACCT	GGATTCCATTCGGGACACCT
	NRXN2	AGAACCAGGCATCCCTCCCT	AGATGCCGGCTTCCCTCAG
	SELL	ACTGGGATTGAAGAAACCACCTG	CCAGGGGATGGCTACAGTTCA
	SIGLEC1	CTCTGTCACCTTCAACAGCCAGA	TGGGGGCATACTTCACTTGGA
	CADM3	ACCCTCAATGTTAATGACCCCA	AGGAAGACAATGAAAGCCACG
	MPZL1	GCTTGGGCTCTTGACAGCT	GTCAACCCGCCAGTCGTA
	SPN	CCTTGGGGTGCTGGTGGTA	GAGGTAGGGCTGAGGTCTGGTC
	CLDN10	TCATACTGTCAGGGCTGTGCTC	CTCCTGCCCATCCAATAAACA
	ICAM3	CGAGTCCTGTATGGTCCCAAAAT	CGTGTCTCGTTTTATCTTTCCATT
	ITGB7	CTCTGCGGAGGCTTTGGT	ACCATAGTAGCCGTCCAAGCA
	GAPDH	TTGGCTACAGCAACAGGGTG	GGGGAGATTCAGTGTGGTGG

antibodies (Abcam, Cambridge, MA), which are against eight proteins that are involved in the ECM-A pathway and are differentially expressed in sPTB, were diluted to 1:1,000, and the peroxidase-conjugated secondary antibody (Abcom), to 1:5,000. The bands were detected by chemiluminescence signals by using ECL Western Blotting Substrate (Thermo Fisher) and scanned to produce digital images. GAPDH served as an internal control.

Statistical analysis

For quantification of RNAs, ΔCt = Ct*target* - Ct*reference* was computed for each sample. ΔCt values, surrogates for RNA abundance in each sample, were compared between groups using an unpaired, two-tailed *t*-test. Differences in DEP were considered significant at $P < 0.05$. The final result was finally reported as relative expression by setting the expression value in the control group as "1". The expression value in the case group was calculated in relation to the control group. All data were given in terms of relative expression of mean ± SD. The data were subjected to one-way ANOVA, followed by an unpaired, two-tailed *t*-test.

RESULTS

Human placental incRNAs associated with ECM-A pathway

The original transcriptomic profile with identification of differentially expressed lncRNAs was deposited in Gene Expression Omnibus with an accession number GSE 50879 (Available from: http://www.ncbi.nlm.nih.gov/geo/info/linking.html). Transcriptomic analysis of chorionic villi from 40 human placenta samples (10 from each subgroup) showed that significant up-regulation of lncADAM9 in pPROM and sPTL as compared to samples from either FTB or PROM [Figure 1].

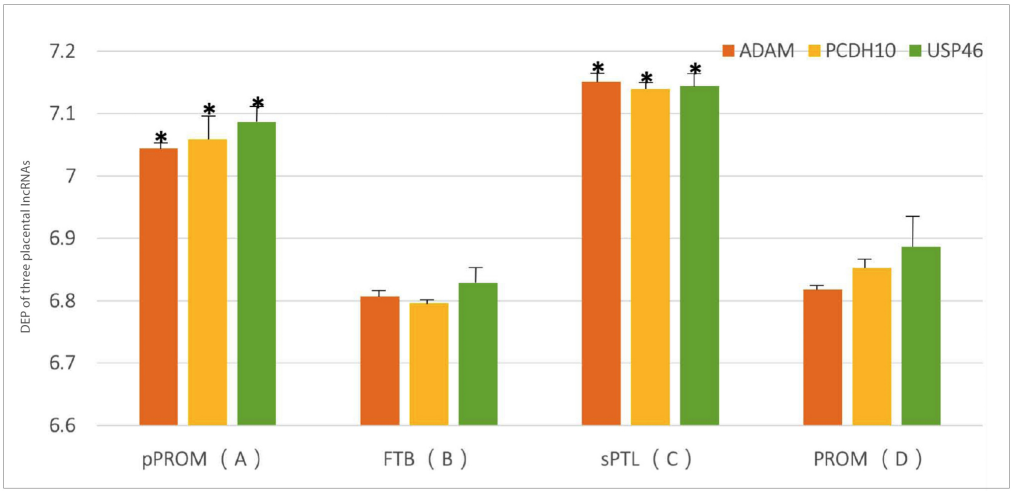

Figure 1. DEP of three placental lncRNAs, the lncADAM9 (orange), lncPCDH10 (yellow), and lncUPS46 (green). Data were collected from 40 pregnancies, including 10 from each pPROM (A), FTB (B), sPTL (C), and PROM (D). The lncRNAs were significantly (*$P < 0.05$) up-regulated in preterm birth (pPROM and sPTL) compared to full-term birth (FTB and PROM), suggesting that lncRNAs may have played epigenetic regulatory roles in preterm syndrome.

Transgenic cell model to overexpress lncADAM9

Based on the results that lncADAM9 was up-regulated in human pregnancies of pPROM and sPTL, *in vitro* study with lncRNA-TG- cultured HTR8 cells for overexpression of lncADAM9 was performed to address whether lncADAM9 may affect global cellular transcription through regulation of its overlapped mRNA-ADAM9. Our results clearly showed a significant down-regulation of the transcription of ADAM9 mRNA [Figure 2C] under the condition of overexpression of exogenous lncADAM9 [Figure 2A]. However, the mRNA of PCDH10 [Figure 2D] was confirmed to not be significantly overexpressed in HTR8 cells in which the lncPCDH10 was transfected [Figure 2B].

Epigenetic impact of lncADAM9 on biological pathways

To better understand the epigenetic regulation of lncRNA beyond the transcription of lncRNA-overlapped mRNAs, we conducted a transcriptomic study with RNA-seq to analyze the global impact of lncRNA. Through Gene Ontology (GO) analysis, transcripts with a top-20 score of up- or down-regulated mRNAs were identified among thousands of transcripts that were impacted by the overexpression of lncADAM9 [Figure 3]. Our results provided evidence that lncADAM9 not only regulates its overlapped ADAM9 transcription, likely through *cis* regulation, but also may have an impact on global transcripts, including the ECM-A pathway [Table 2], although the underlying mechanism is yet unknown. Briefly, a total of 8,354 differentially expressed genes (DEGs) were detected in the group of lncADAM9 overexpression compared to their blank control, and the corresponding genes were enriched into four pathways and 23 GO entries on ECM and adhesion. A total of 7,795 differentially expressed genes were detected in the group of lncPCDH10 overexpression cells compared to their blank controls, and the corresponding genes were enriched into seven pathways and 14 GO entries associated with adhesion and ECM [Table 2]. The DEGs of lncADAM9-TG and lncPCDH10-TG were enriched on the same GO entries, including ECM structural constituent, proteinaceous extracellular matrix, homophilic cell adhesion *via* plasma membrane adhesion molecules, leukocyte cell-cell adhesion, biological adhesion, ECM organization, homotypic cell-cell adhesion, single organism cell adhesion, and cell-cell adhesion *via* plasma-membrane adhesion molecules. The common pathway shared between lncADAM9-TG and lncPCDH10-TG was cell adhesion molecules. These results indicated that overexpressed lncADAM9 and lncPCDH10 influence ECM and cell adhesion (ECM-A), and their common pathway is cell adhesion-related molecules.

Table 2. Pathways impacted by transgenic lncADAM9 and lncPCDH10

Transgenic	Pathway/GO ID	Pathway/GO name	Annotated	Significance	FDR
LncADAM9	hsa04610	Complement and coagulation cascades	79	23	1.00E-03
	hsa04514	Cell adhesion molecules (CAMs)	141	26	1.71E-01
	hsa04915	Estrogen signaling pathway	98	19	2.19E-01
	hsa04611	Platelet activation	123	21	3.90E-01
	GO:0007155	cell adhesion	1425	223	1.28E-05
	GO:0022610	biological adhesion	1432	223	1.81E-05
	GO:0098742	cell-cell adhesion via plasma-membrane adhesion molecules	235	49	0.00151
	GO:0007156	homophilic cell adhesion via plasma membrane adhesion molecules	156	35	0.00372
	GO:0016337	single organismal cell-cell adhesion	859	131	0.00652
	GO:0098609	cell-cell adhesion	859	131	0.00652
	GO:0098602	single organism cell adhesion	914	135	0.01637
	GO:0005201	extracellular matrix structural constituent	78	19	0.02893
	GO:0005578	proteinaceous extracellular matrix	399	66	0.03486
	GO:0034116	positive regulation of heterotypic cell-cell adhesion	12	5	0.19202
	GO:0045785	positive regulation of cell adhesion	375	57	0.2104
	GO:0022407	regulation of cell-cell adhesion	393	59	0.23413
	GO:0022409	positive regulation of cell-cell adhesion	241	39	0.24343
	GO:0034109	homotypic cell-cell adhesion	529	76	0.24879
	GO:0007159	leukocyte cell-cell adhesion	490	71	0.25472
	GO:0034110	regulation of homotypic cell-cell adhesion	329	50	0.25472
	GO:0030198	extracellular matrix organization	338	51	0.2677
	GO:0030155	regulation of cell adhesion	645	89	0.30233
	GO:1903037	regulation of leukocyte cell-cell adhesion	311	47	0.30601
	GO:0034111	negative regulation of homotypic cell-cell adhesion	109	19	0.4138
	GO:0044420	extracellular matrix component	183	29	0.42227
	GO:1903039	positive regulation of leukocyte cell-cell adhesion	208	32	0.42413
	GO:0034112	positive regulation of homotypic cell-cell adhesion	211	32	0.46612
LncPCDH10	hsa04514	Cell adhesion molecules (CAMs)	141	31	2.63E-03
	hsa04670	Leukocyte transendothelial migration	112	22	1.33E-01
	hsa04512	ECM-receptor interaction	82	16	2.11E-01
	hsa05144	Malaria	49	11	2.11E-01
	hsa04064	NF-kappa B signaling pathway	93	16	3.65E-01
	hsa05322	Systemic lupus erythematosus	130	20	4.78E-01
	GO:0022610	biological adhesion	1432	203	1.80E-05
	GO:0007155	cell adhesion	1425	202	1.80E-05
	GO:0098742	cell-cell adhesion via plasma-membrane adhesion molecules	235	51	2.79E-05
	GO:0016337	single organismal cell-cell adhesion	859	125	0.00129
	GO:0098609	cell-cell adhesion	859	125	0.00129
	GO:0007156	homophilic cell adhesion via plasma membrane adhesion molecules	156	34	0.00187
	GO:0016338	calcium-independent cell-cell adhesion via plasma membrane cell-adhesion molecules	23	10	0.00722
	GO:0098602	single organism cell adhesion	914	127	0.00724
	GO:0005578	proteinaceous extracellular matrix	399	56	0.14638
	GO:0030198	extracellular matrix organization	338	48	0.23419
	GO:0005201	extracellular matrix structural constituent	78	14	0.35866
	GO:0031012	extracellular matrix	584	73	0.39601
	GO:0007159	leukocyte cell-cell adhesion	490	62	0.41435
	GO:0034109	homotypic cell-cell adhesion	529	65	0.47199

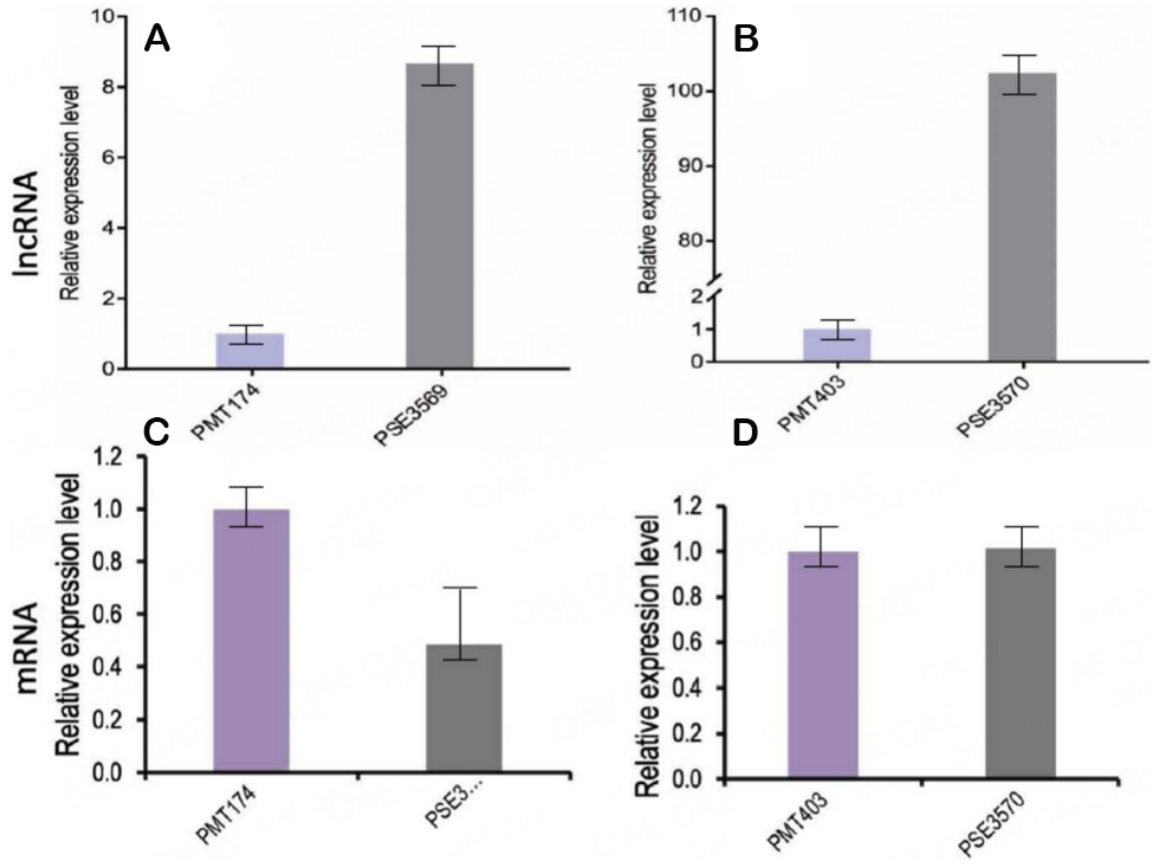

Figure 2. Impact of over-expressed exogenous lncRNA of ADAM9 (A) and PCDH10 (B) on their overlapped mRNA (C and D), respectively. PSE presents cells transfected with plasmids carrying inserts. PMT presents cells transfected with an empty vector. Results were obtained from at least duplicated experiments.

DEPs of ECM-A in human fetal membranes

Transgenic cell culture is an *in vitro* condition in which the transcriptomic profiles impacted by overexpressed lncRNAs may not be present in primary tissues that may more accurately reflect the intrauterine environment. Quantitative measurement in human fetal membranes from each subgroup of sPTL, pPROM, PROM, and FTB confirmed transcripts (CNTN1, NRXN2, SPN, ICAM2, ICAM3, CADM1, CADM3, HLA-DPB1, MPZL1, SIGLEC1, CD274, CLDN7, CLDN10, ITGAL, SELL, ITGB7) of the ECM-A pathway in variant outcomes of pregnancies. The DEPs of mRNA at CNTN1, NRXN2, CADM1, HLA-DPB1, ICAM2, and SPN in sPTL were significantly up-regulated [Figure 4], which provided strong evidence that the ECM-A pathway is associated with sPTL. Furthermore, Western blots [Figure 5] showed variant expression patterns of ECM-A proteins in preterm birth (sPTL and pPROM) *vs.* full-term birth (FTB and PROM). Generally, ICAM2, CADM1, CADM3, SPN, CNTN1, and HLA-DPB1 were up-regulated, but NRXN2 was down-regulated in sPTL compared to FTB (c *vs.* b). When pPROM was compared to PROM (a *vs.* d), the protein signals of ICAM2, CADM1, CADM3, SPN, CNTN1, and HLA-DPB1 were reduced.

Interaction network of cell adhesion molecules

ECM-A molecules were subjected to analysis of the interaction network. As shown in Figure 6, two intensive co-expression groups containing the nine CAMs were present in an interaction network connected by PTPRZ1 and ITGB2. The gene products of CADM1, CADM3, NRXN2, CNTN1, and MZPL1 formed a co-expression group that is localized at different subcellular compartments, four of which, the CADM1, CADM3, NRXN2, and CNTN1, were highly correlated with sPTL. CADM1 was highly expressed

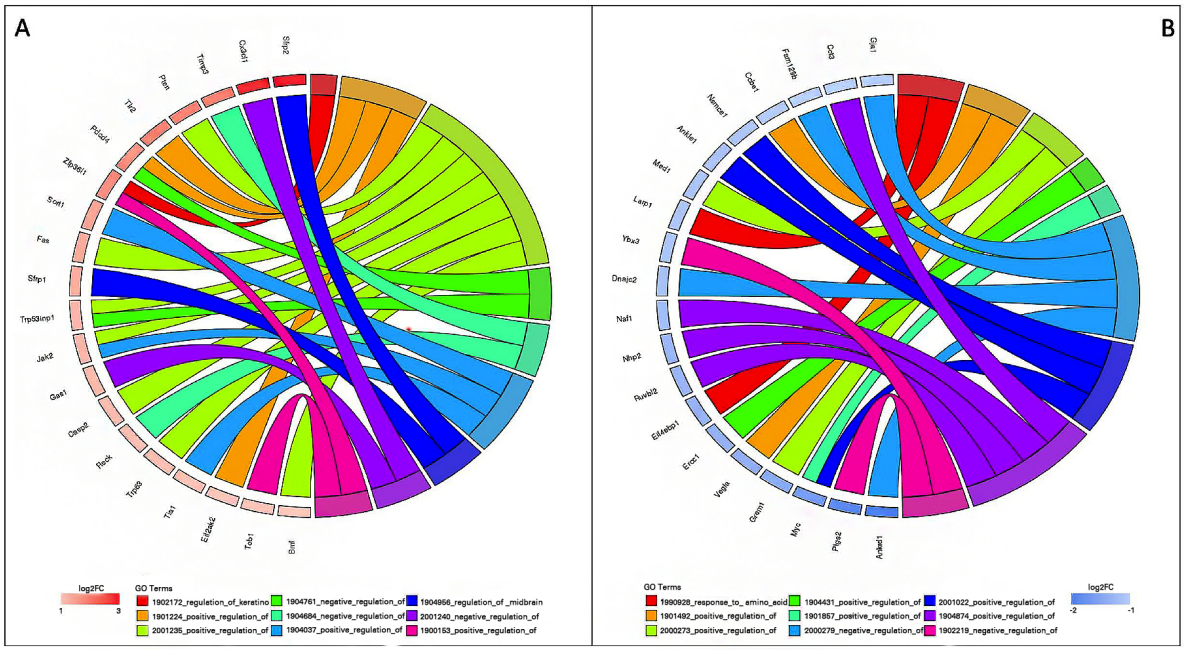

Figure 3. Transcriptomic profiles in lncADAM9-transgenic HTR8 cells. Epigenetic impact of overexpressed lncADAM9 transfected in HTR8 cells resulted in a global differential expression of mRNAs. Top-scored 20 transcripts, either up-regulated (A) or down-regulated (B), were determined with GO analysis.

in epithelial cells. Another group of co-expressed gene products consisted of ICAM2, SPN, SIGLEC1, and HLA-DPB1, which were usually expressed in immune cells.

DISCUSSION

To investigate the role of lncRNAs in a potential epigenetic mechanism underlying sPTB, we identified DEPs of lncRNAs in placental samples affected by sPTB[3]. We undertook a transcriptome analysis with *ex vivo* and *in vitro* functional studies, focusing on the pathogenic impact of lncADAM9 on the ECM-A pathway in sPTB. These were confirmed by the observation of differentially expressed ECM-A-associated mRNAs and proteins in fetal membranes of sPTB and lncADAM9-TG-cells in culture. Our findings provided evidence of the involvement of the ECM-A pathway in the pathogenesis of sPTB.

Epigenetic regulation of lncRNA in sPTB

The high heterogeneity of sPTB is associated with its multifactorial etiology, whereby gene-environmental interactions are recognized as significant contributors. Aberrant transcription of lncRNA has been found in the uterus and placenta of women who have experienced premature birth[3,30,31]. We have performed a series of studies to discover and verify the regulation of lncRNA in sPTB. Thousands of differentially expressed lncRNAs were identified from sPTB placentas, providing evidence of lncRNAs' involvement in the pathogenic process of sPTB. Furthermore, functional pathways were enriched to be associated with infection and inflammatory response, ECM-receptor interactions, cell adhesion molecules, and smooth muscle contraction in sPTB[3,32,33], suggesting that epigenetic regulation of lncRNAs may be involved in these pathways. The co-differentially expressed pairs of lncRNAs and mRNAs sharing the same genomic loci in sPTB were further identified as being related to the UPS and infection-inflammation pathways[22]. The infection-inflammation pathways have been proven to be major causes of preterm birth[18,34]. Collagen degradation reduces the physical strength of the fetal membranes and contributes to pPROM. Other studies have found that differential expression of lncRNAs in the cervix and myometrium is associated with the length of gestation[30,31]. Evidence for the epigenetic regulatory role of lncRNA in preterm labor has been

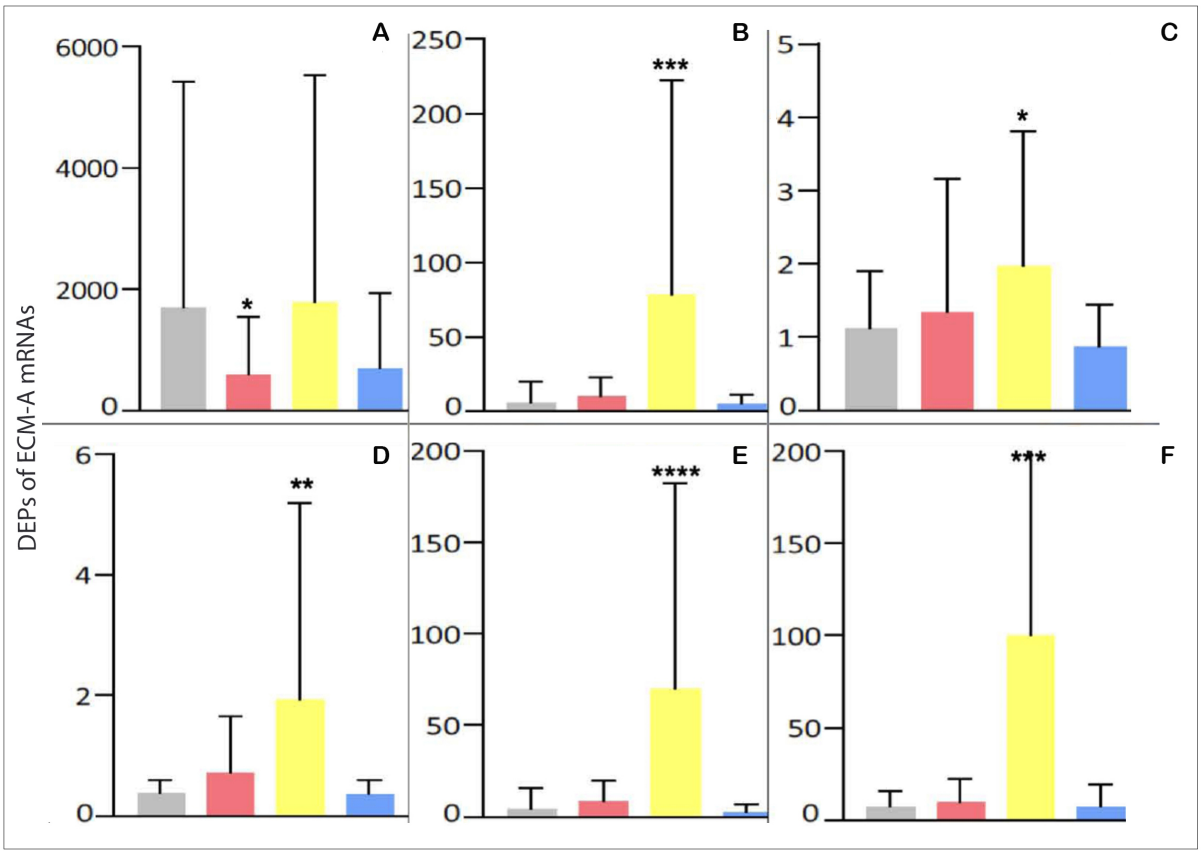

Figure 4. DEPs of ECM-A mRNAs were quantified with human fetal membranes of pPROM (gray), sPTL (red), FTB (yellow), and PROM (blue). Quantitative measurement of mRNAs, which are involved in the ECM-A pathway, includes (A) CADM1 ($P = 0.025$), (B) CNTN1 ($P = 0.0002$), (C) HLA-DPB1 ($P = 0.0178$), (D) ICAM2 ($P = 0.0013$), (E) NRXN2 ($P < 0.0001$), (F) SPN ($P = 0.0002$). Statistical significance of differential expression, such as sPTL *vs.* pPROM or sPTL *vs.* FTB (A) and pPROM *vs.* FTB or sPTL *vs.* FTB (D-F) has been observed with $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$, $^{****}P < 0.0001$.

provided, and this has strengthened our confidence in seeking to explore the pathogenesis of sPTB with further examination of lncRNA.

LncADAM9 regulatory function in sPTB

LncRNA-regulated transcripts (the lncRNA-overlapped mRNAs) include nine pairs of differentially expressed CUP-lncRNAs -mRNAs that were identified in our previous studies of human placentas[3,22,23]. LncADAM9 (NR_027638), the focus of this study, is a sense transcript, whose sequence is 96% identical to mRNA-ADAM9. The transcript of lncADAM9 was found to be increased while the expression of ADAM9-mRNA was down-regulated in human placentas of sPTL and pPROM, compared to full-term births. Many pairs of lncRNA-mRNA from the same strands have been found to show opposite expression trends, considered as epigenetic trans regulation[35]. The altered expression pattern of lncRNA/mRNA illustrated that lncADAM9 may have a trans regulation on the transcription of mRNA-ADAM9, although the detailed regulatory mechanism needs further investigation.

Environmental factors, such as osmotic and oxidative stresses, have been determined to induce a metalloprotease activity leading to cell surface cleavage of pro-heparin-binding EGF (pro-HB-EGF) and subsequent EGFR activation. This ligand-dependent EGFR signal resulted from stress-induced activation of the MAPK p38 in human carcinoma cells and was mediated by the metalloproteases ADAM9[36]. A

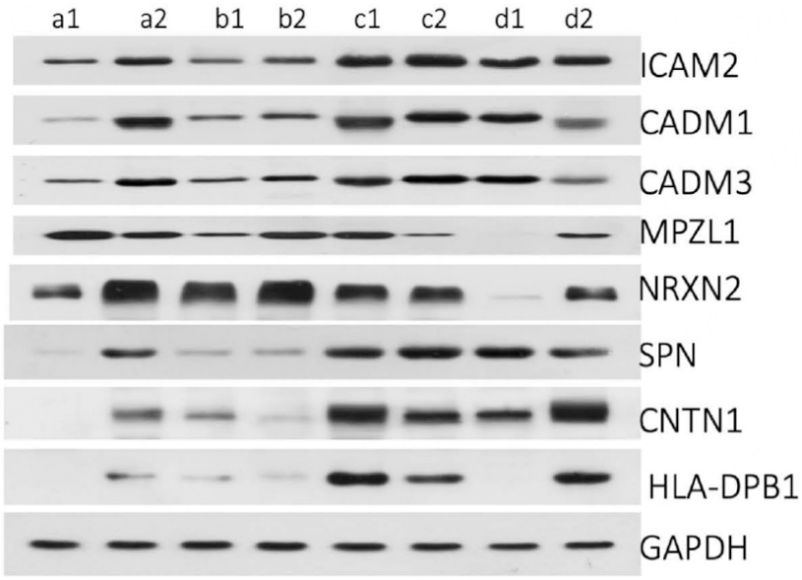

Figure 5. Western blots of ECM-A proteins. Protein samples were isolated from two unrelated human fetal membrane samples (1, 2), which were delivered from various outcomes of pregnancies, including (a) pPROM, (b) FTB, (c) sPTL, and (d) PROM. Antibodies against the ECM-A pathway were used to probe ECM-A proteins. GAPDH was used as an internal control for protein loading.

functional domain, the metalloproteinase domain, of ADAMs is similar to the matrix metalloproteases (MMPs), which include MMP-1, MMP-8, and MMP-9 and have been reported to be involved in the fetal membrane rupture[37-40]. The proteases MMP-8 and MMP-9 involved in ECM degradation were elevated in the amniotic fluid, which may reduce the mechanical support of the membrane. MMPs are the major proteases involved in ECM degradation[41]. They can degrade ECM protein substrates, including collagens, fibronectin, laminin, and vitronectin, by breaking the cell-to-cell and cell-to-ECM adhesion. In fact, the amount of ADAM8 was found to be elevated in the amniotic fluid of women who gave birth prematurely, which is thought to be a possible risk factor for preterm birth[42]. Therefore, it is likely that ADAM9 is involved in the metabolism of the ECM and thus in preterm birth.

ECM-A and sPTB

The ECM plays an active and dynamic role that both reflects and facilitates the functional requirements of a tissue. Our earlier study determined that ECM proteins COL4A1, LAMA2, FBLN2, and APMAP were downregulated in the premature tissues of human placentas, including fetal membranes in spontaneous preterm births[43]. Applying experimental models may further address the contribution of the interaction between the intrauterine environment and gene expression on the pathogenic mechanism(s) of sPTB[22,23]. Studies on mice deficient in the proteoglycan decorin have demonstrated that progesterone and estrogen may regulate ECM organization and turnover, expressions of factors required for assembly and synthesis of collagen and elastic fibers are temporally regulated, and the ultrastructure of collagen fibrils and elastic fibers is markedly altered during pregnancy in wild-type mice[44].

Our results in this study certainly supported that any possible environmental factors that result in the alteration of lnc-ADAM9 may epigenetically regulate mRNA-ADAM9 and consequently has an impact on the ECM-A pathway in the sPTB. In the current study, to better understand the epigenetic regulation of lncADAM9 on its overlapped mRNAs, lncRNA-TG HTR8 cells were constructed, in which the *in vivo* expression pattern of lncADAM9 and its mRNA simulated *in vitro*, with lncADAM9 up-regulated and mRNA-ADAM9 down-regulated. Many transcripts were impacted by overexpressed lncADAM9 in the

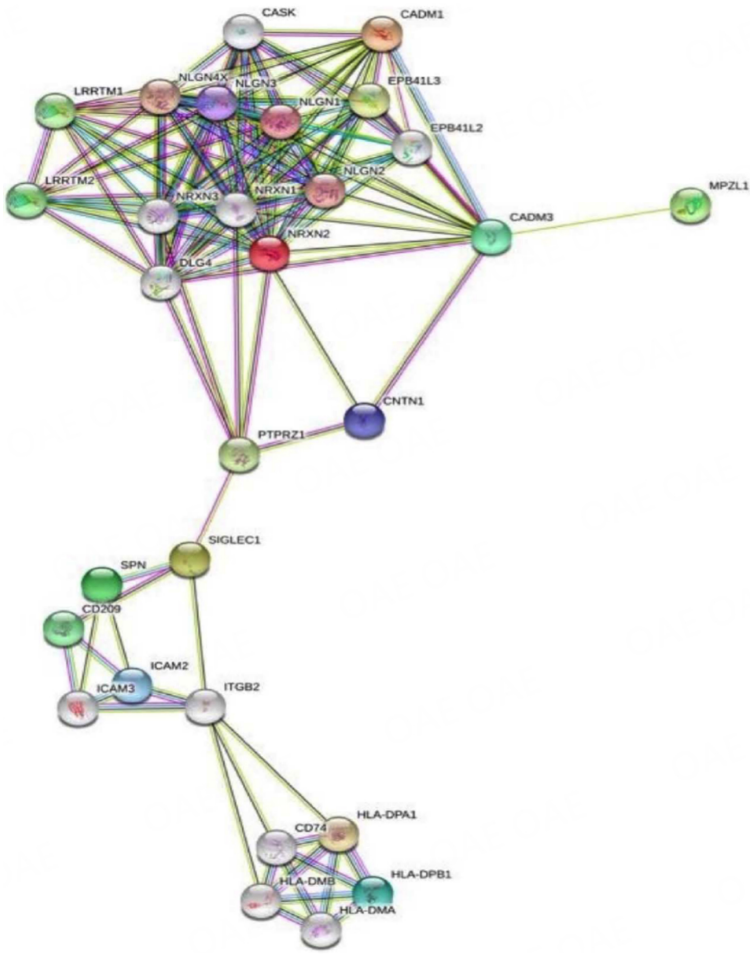

Figure 6. ECM-A protein interactomics map. Two intensive co-expression groups containing the nine CAMs were present in an interaction network connected by PTPRZ1 and ITGB2. The genes of CADM1, CADM3, NRXN2, CNTN1, and MZPL1 formed a co-expression group, four of which happened to be highly correlated with sPTL. KEGG pathway showed that CADM1, CADM3, NRXN2, and CNTN1 were expressed in different subcellular compartments. ICAM2, SPN, SIGLEC1, and HLA-DPB1 are usually co-expressed in immune cells.

transgenic HTR8 cells; however, we focused on the molecules associated with the ECM-A pathway to address how lncRNA might regulate preterm labor, specifically *via* the ECM-A pathway. Evidence obtained in our transcriptome analysis indicates that lncADAM9 is likely to regulate the expression pattern of the ECM-A pathway. Transcripts and proteins of CNTN1, CADM1, CADM3, NRXN2, HLA-DPB1, and ICAM2 [Figures 4 and 5], suggesting ECM-A degradation has been proved to be associated with sPTB, partically pPROM[32,33].

Few of the molecules uncovered by our analysis have been reported in previous studies about preterm labor. CNTN1[45], NRXN2[46], CADM1[47], and CADM3[48] are molecules related to the nervous system; MPZL1 has been reported to promote tumor cell proliferation and migration in hepatocellular carcinoma and ovarian cancer[49,50]; and ICAM2[51] and HLA-DPB1 are molecules related to antigen recognition specificity in the immune response. Our findings, obtained initially from human placentas and followed by fetal membranes, suggested that the ECM-adhesion pathway we studied may play a critical role in placental development by mediating cell recognition and adhesion and may mediate intracellular signaling.

In conclusions, Transcriptomic and functional analysis with transgenic human chorionic trophoblast cell modeling, and verification by human fetal membranes determined that lncADAM9 is an epigenetic regulator that is up-regulated in placentas of sPTL and pPROM. The up-regulated lncADAM9 may decrease the gene transcription of mRNA-ADAM9 and have a pathogenic impact on the ECM-A pathway in sPTB. The differentially expressed ECM-A molecules between sPTL and pPROM may have the potential to be applied as a novel biomarker to distinguish sPTL from pPROM at an early stage of pregnancy.

DECLARATIONS

Authors' contributions

Conceptualized and conceived the research project; consolidated research funds: Zhong N, Wang G
Designed, oversaw, and supervised the detailed studies: Zhong N
Conducted the studies: Wang X, Chu C, Dong X
Performed specimen collection: Wang G, Wang X, Liu A, Hou D, Zhang J, Jia Y, Yang X, Ji Y, Wang T
Analyzed the research data: Wang G, Wang X, Hou D, Dong X, Ju W, Bonney EA, Zhong N
Drafted the manuscript: Hou D, Dong X, Ju W, Bonney EA, Zhong N
Finalized the manuscript and submitted the manuscript on behalf of all authors: Zhong N
All authors reviewed and approved the manuscript.

Availability of data and materials

All materials and data were previously coded and are anonymous to the authors of this study. The transcriptomic data have been deposited in Gene Expression Omnibus with an accession number GSE 50879.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The study design was reviewed and approved by the Ethics Committee of Inner Mongolia Maternal and Child Health Care Hospital. Written informed consent was obtained from the pregnant women who participated in this study.

Consent for publication

Not applicable.

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