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Original Paper

Analysis of Long Noncoding RNA Expression Profile in Human Pulmonary Microvascular Endothelial Cells Exposed to Lipopolysaccharide

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Key Words
Long noncoding RNA • Expression profile • Acute lung injury • Lipopolysaccharide • Human pulmonary microvascular endothelial cell

Abstract
Background/Aims: Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are a continuum of life-threatening lung changes. Pulmonary vascular injury is one of the most important initial causes of ALI and ARDS. However, the functions of long noncoding RNAs (lncRNAs) in pulmonary endothelial injury remain largely unknown. The aim of the present study was to determine the lncRNA expression profile of human pulmonary microvascular endothelial cells (HPMECs) exposed to lipopolysaccharide (LPS) and explore the potential functions of differentially expressed lncRNAs. ***Methods:*** Microarray analysis was used to identify differentially expressed lncRNAs and mRNAs. Bioinformatics analyses, including Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, lncRNA-mRNA coexpression network and transcription factor (TF)-lncRNA network analyses, were performed to predict the functions of significantly differentially expressed lncRNAs and mRNAs. Real-time polymerase chain reaction (PCR) was used to determine the expression of selected lncRNAs and mRNAs. ***Results:*** In this study, we found that 213 lncRNAs and 212 mRNAs were significantly differentially expressed in HPMECs exposed to LPS (*fold change* > 2.0, $p < 0.05$). Furthermore, we found that mRNAs co-expressed with lncRNAs were significantly enriched in the TNF signaling pathway, the NF-κB signaling pathway, cell adhesion molecules (CAMs), cytokine-cytokine receptor interactions, and extracellular matrix (ECM)-receptor interactions. The expression levels of all but one of the selected lncRNAs and mRNAs detected by real-time PCR were similar to those detected by microarray analysis. ***Conclusion:*** Our data indicate that lncRNAs play an important role in LPS-induced pulmonary endothelial inflammation and barrier dysfunction and may be potential preventive and therapeutic targets for ALI and ARDS.

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Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are part of a continuum of life-threatening lung changes that interfere with the diffusion of oxygen from alveoli into the blood [1-4]. Sepsis and pneumonia are the most common causes of ARDS, and pulmonary vascular injury is one of the most important initial causes of ALI and ARDS. ARDS accounts for 10% of intensive care unit admissions globally. Despite years of basic and clinical studies, the global mortality of ARDS has been as high as 40% in recent years [5, 6]. For these reasons, the identification of key molecules involved in ARDS is highly demanded for improving the clinical outcome of this syndrome.

It is now generally accepted that the majority of mammalian RNA transcripts are noncoding RNAs (ncRNAs). Long noncoding RNAs (lncRNAs), which are longer than 200 nucleotides, are generally not translated into proteins [7-10]. By regulating gene expression at posttranscriptional, transcriptional and epigenetic stages, lncRNAs participate in most essential biological processes. Over the past decade, the study of lncRNAs has become a hot spot in basic and clinical research of ALI and ARDS. Recent studies have shown that miRNAs are involved in the pathophysiology of ARDS and may be interesting diagnostic biomarkers and therapeutic targets [11-20]. However, less is known about the role of lncRNAs in the pathogenesis of ALI and ARDS.

To study the function of lncRNAs in the pathogenesis of ALI and ARDS, we established an experimental model of pulmonary endothelial inflammation and barrier dysfunction by stimulating HPMECs with LPS [21-24] and investigated the expression profile of lncRNAs and mRNAs by microarray analysis. We found that 213 lncRNAs and 212 mRNAs were significantly differentially expressed. Bioinformatics analyses indicated that the differentially expressed lncRNAs might play an important role in LPS-induced pulmonary endothelial inflammation and barrier dysfunction.

These findings will serve to increase the understanding of the pathogenesis of pulmonary endothelial dysfunction. Moreover, due to opportunities to identify novel therapeutic and preventive targets, our results may provide relevant information for future clinical interventions of ALI and ARDS.

Materials and Methods

Cell culture, LPS treatment, and RNA isolation

Human pulmonary microvascular endothelial cells (HPMECs, ScienCell, San Diego, CA, USA) were cultured in endothelial cell medium (ECM, ScienCell, San Diego, CA, USA) in a humidified 5% CO_2 incubator at 37°C. LPS (Sigma, St Louis, MO, USA) from Escherichia coli O111:B4 was dissolved in sterile water and prepared fresh at the time of use. At approximately 90% confluence in culture, HPMECs were starved for 1 hour (h) in serum-free medium and then stimulated for 4 h with LPS (1 μg/ml) or vehicle control (PBS) in ECM containing 1% FBS as previously described [14] [21] [25]. HPMECs were used in passage numbers 4 to 6. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quality of the RNA preparations was verified on an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Microarray analysis

Total RNA was hybridized to Affymetrix Human Transcriptome Array 2.0 (Affymetrix, Santa Clara, CA, USA). Hybridized data were preprocessed and statistically analyzed as described previously [26, 27]. Differentially expressed lncRNAs and mRNAs were identified by fold-change screening at a threshold of 2.0-fold or greater and a p value < 0.05.

GO and KEGG pathway analyses

GO analysis (http://www.geneontology.org) was performed to explore functions of genes based on the biological pathway, cellular component and molecular function categories; Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) analysis was performed to determine pathways significantly enriched in genes. $P < 0.05$ and FDR < 0.05 were used as thresholds to define significantly enriched GO terms or pathways.

Table 1. Primers designed for real-time PCR of candidate lncRNAs and mRNAs. Tm: temperature. bp: base pair

Primer name	Primer FW (5'-3')	Primer RW (5'-3')	Tm	Product length
n384765	AGATGGAAAGGGGTGCTTGG	GCAGAGTGTGAGAAGGGTGG	60	119
n344917	ACTCCAAGGCGTTTTTCCAC	GGAAGCCTCGACCCTGTATTG	60	104
XLOC_l2_015215	GCTCATCACACAGAACTTTTCTCA	AGAACAAAATACTCTGGGGAGAAG	60	103
n340107	GTCCTCCTCATCTTCCTCTTCC	ACAGGCTCATCAGTTAGCATCT	60	100
n407205	GAGGAACACTGGGTTGGACT	TGCCACCCTGGGGAATATAAAG	60	117
NR_001564	ATTTCTTACTCTCTCGGGGCTG	CCATAAAGGGTGTTGGGGGA	60	100
SELE	AGCCTTCAGTGTACCTCATCT	GCACCTCACAGAGCCATTC	60	105
IL8	TTCTAGGACAAGAGCCAGGAAG	ATCAGGAAGGCTGCCAAGAG	60	102
VCAM1	ATACCCTCCCAGGCACACA	CTCCAAGGATCACGACCATCT	60	111
ICAM1	ATGCCCAGACATCTGTGTCC	GGGGTCTCTATGCCCAACAA	60	112
CXCL10	ATATGGCACACTAGCCCCAC	GATTCATGGTGCTGAGACTGGA	60	100
MMP10	TTACATTGCTAGGCGAGATAGG	CAGTCACAGAACATGCAGGAA	60	120
ABI3BP	GGCAGCAATGTATCACCAAAC	GAGCAGGTCGCACAACTATC	60	109
TRPC6	AGGATGACGCTGATGTGGAG	GGACTCGGCACCAGATTGA	60	106

Real-time PCR

A FastQuant RT Kit (Tiangen, Beijing, China) was used to reverse transcribe total RNA into cDNA following the manufacturer's directions. Real-time PCR was performed using SuperReal PreMix Plus (SYBR Green) (Tiangen, Beijing, China) in the Applied Biosystems GeneAmp® PCR System 9700. The reaction conditions were as follows: incubation at 95°C for 15 min, followed by 40 cycles of 95°C for 10 s and 60°C for 20 s. The relative expression levels of lncRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH levels [28]. The primers for each lncRNA and mRNA are listed in Table 1.

LncRNA-mRNA coexpression network

A lncRNA-mRNA coexpression network was constructed to identify the interactions between lncRNA and mRNA according to the normalized signal intensity of specific mRNA and lncRNA expression levels as described previously [29, 30].

TF-lncRNA network

Transcription factors (TFs) and DNA sequence motifs from 2.0 kilobase upstream of the transcription start site of differentially expressed lncRNAs were predicted with the TRANSFACT professional database (http://gene-regulation.com/). TFs with a matrix score and core score equal to 1 were selected.

Statistical analysis

All data are expressed as the mean ± SEM. For comparisons between 2 groups, unpaired Student's t-test for parametric data and Mann-Whitney's U-test for nonparametric data were used. All statistical analyses were performed with GraphPad Prism 7.04 (GraphPad Software, San Diego, CA, USA). A p value < 0.05 was considered statistically significant.

Results

Profiles of the differentially expressed lncRNAs and mRNAs

To evaluate the differential expression of lncRNAs in HPMECs stimulated with LPS, we performed microarray analysis of the lncRNA expression profile using Affymetrix Human Transcriptome Array 2.0, which covers 245, 349 coding transcripts and 40, 914 noncoding transcripts.

We found that 213 lncRNAs were significantly differentially expressed (*fold change* > 2.0, $p < 0.05$). Of these, 189 lncRNAs were upregulated and 28 lncRNAs were downregulated (Fig. 1A, Fig. 1C). The top 20 most significantly upregulated (Table 2) and downregulated (Table 3) lncRNAs are listed below. At the same time, 212 mRNAs were significantly differentially expressed (*fold change* > 2.0, $p < 0.05$), including 183 upregulated mRNAs

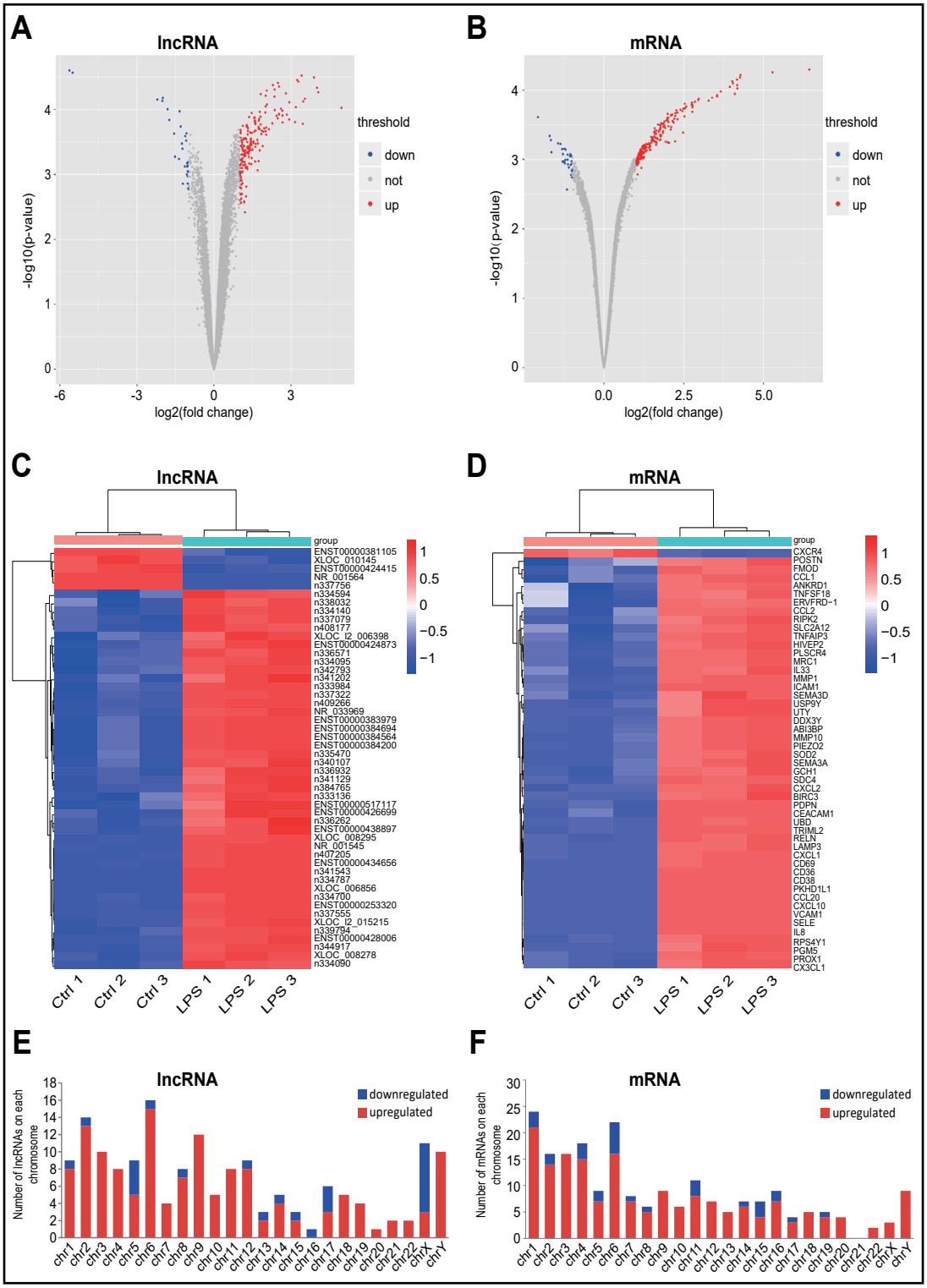

Fig. 1. Volcano plots, expression profiles and chromosome distribution of differentially expressed lncRNAs and mRNAs in HPMECs treated with LPS. Volcano plots of differentially expressed lncRNAs (A) and mRNAs (B). Gray dots indicate no change. Blue and red dots indicate significantly downregulated and upregulated lncRNAs and mRNAs, respectively. Hierarchical clustering indicates lncRNA (C) and mRNA (D) profiles. Red and blue columns refer to high and low relative expression, respectively. Distribution of differentially expressed lncRNAs (E) and mRNAs (F).

Table 2. Top 20 significantly upregulated lncRNAs in HPMECs treated with LPS. Fold: fold change. Chr: chromosome number. LPS 1 to 3 and Ctrl 1 to 3: normalized gene signals of each sample

Accession	P value	Fold	Chr	Gene start	Gene end	LPS1	LPS2	LPS3	Ctrl	Ctrl	Ctrl
n409266	0.00003	10.6	chr3	14591012	14596896	10.4	10.5	10.6	7.30	7.00	7.20
n337322	0.00003	14.8	chr1	10266777	10266807	11.3	11.3	11.3	7.65	7.14	7.51
XLOC_008278	0.00003	9.43	chrY	3904538	3968361	8.13	8.25	8.07	4.67	5.05	5.02
n334140	0.00003	9.76	chr1	43013360	43025500	10.0	9.86	10.0	6.43	6.92	6.65
XLOC_l2_015215	0.00004	4.89	chr9	68773916	68778906	7.14	7.28	7.36	5.00	4.91	5.00
NR_001545	0.00004	5.77	chrY	14774265	14804162	5.30	5.36	5.56	2.79	2.96	2.88
n334090	0.00004	5.13	chr3	13923725	13925844	8.67	8.56	8.47	6.11	6.17	6.35
n344917	0.00005	4.16	chr6	86386800	86388112	5.80	5.92	5.95	3.78	3.81	3.92
XLOC_006856	0.00005	16.6	chr8	79854913	79855506	7.06	7.92	7.67	3.62	3.45	3.41
n407205	0.00005	6.95	chr1	9906735	9913497	5.68	5.78	5.96	3.04	2.81	3.19
ENST000004359	0.00005	3.52	chr6	74779167	75400443	5.12	5.09	5.08	3.35	3.30	3.20
n339794	0.00006	4.03	chr1	10731474	10773583	7.76	7.76	7.84	5.73	5.67	5.93
n408177	0.00006	5.09	chrY	16636454	16955848	6.78	6.60	6.53	4.23	4.48	4.16
ENST000004280	0.00006	11.6	chr9	13446525	13487510	7.47	7.41	7.81	3.84	3.74	4.48
n334095	0.00007	11.0	chr1	10396161	10396374	10.7	10.8	11.0	7.64	6.91	7.63
ENST000004266	0.00007	7.63	chrY	3904538	3968361	7.07	7.63	7.55	4.29	4.70	4.47
ENST000003845	0.00008	4.17	chr1	15244554	15244653	5.69	5.73	5.72	3.86	3.50	3.60
ENST000005179	0.00008	4.02	chr5	15989524	15991470	5.65	5.83	5.96	3.85	3.72	3.86
n337555	0.00009	9.08	chrY	15470990	15471751	6.51	6.76	7.06	3.78	3.23	3.78
n334787	0.00009	31.1	chr4	74608856	74609068	6.47	5.81	7.38	1.28	1.52	1.61

Table 3. Top 20 significantly downregulated lncRNAs in HPMECs treated with LPS. Fold: fold change. Chr: chromosome number. LPS 1 to 3 and Ctrl 1 to 3: normalized gene signals of each sample

Accession	P value	Fold	Chr	Gene start	Gene end	LPS	LPS	LPS	Ctrl	Ctrl2	Ctrl3
NR_001564	0.00002	-	chrX	73040486	73072588	3.24	3.20	3.00	8.71	8.72	8.90
ENST000004244	0.00006	-3.98	chrX	3809479	3838787	5.32	5.44	5.51	7.42	7.31	7.53
ENST000003811	0.00007	-4.61	chrX	3847910	3855896	5.00	4.65	4.70	7.02	6.99	6.95
XLOC_010145	0.00007	-4.04	chr1	88775675	88813029	3.57	3.66	3.46	5.71	5.49	5.52
ENST000003811	0.00009	-3.50	chrX	3771051	3800358	5.68	5.78	5.82	7.54	7.48	7.70
n340852	0.00010	-2.53	chr6	13358067	13361194	6.06	6.05	5.99	7.36	7.39	7.39
XLOC_l2_015669	0.00014	-3.35	chrX	3820107	3855883	5.47	5.36	5.48	7.18	7.02	7.35
NR_039968	0.00018	-2.46	chr5	72174418	72174490	4.09	4.19	4.04	5.30	5.46	5.46
XLOC_008015	0.00023	-2.93	chrX	73070670	73072528	3.49	3.58	4.02	5.28	5.25	5.20
NR_034031	0.00025	-2.18	chrX	3734598	3761935	5.48	5.72	5.68	6.71	6.67	6.86
n335593	0.00030	-2.12	chr1	20944648	20945246	8.87	8.92	8.98	9.83	10.0	10.1
XLOC_l2_006085	0.00033	-2.38	chr1	41426870	41428223	4.35	4.07	4.11	5.20	5.49	5.60
XLOC_l2_015668	0.00040	-2.34	chrX	3782439	3799884	5.49	5.46	5.68	6.59	6.64	7.08
n342704	0.00053	-2.87	chr1	41437409	41438729	4.41	4.09	4.08	5.44	5.45	6.24
n337065	0.00057	-2.58	chr5	69372740	69373356	4.81	5.22	5.55	6.39	6.44	6.84
n334398	0.00064	-2.02	chr1	59479134	59480524	5.55	5.79	5.92	6.78	6.99	6.54
NR_037504	0.00066	-2.07	chr6	16741129	16741140	4.73	4.41	4.24	5.29	5.63	5.59
XLOC_002531	0.00073	-2.04	chr2	23833756	23834346	3.84	3.61	4.23	5.01	4.82	4.92
n336284	0.00074	-2.20	chr1	82761080	82761299	3.14	3.71	3.30	4.31	4.82	4.43
ENST000005072	0.00075	-2.06	chr5	33424131	33440725	4.32	3.76	4.33	5.06	5.27	5.20

and 29 downregulated mRNAs (Fig. 1B, Fig. 1D). The top 20 most significantly upregulated (Table 4) and downregulated (Table 5) mRNAs are listed below.

Although differentially expressed lncRNAs and mRNAs were widely scattered among all chromosomes, the distribution was not equal (Fig. 1E, Fig. 1F). Chromosome 1 and chromosome 6 had the largest number of differentially expressed mRNAs and lncRNAs, respectively. Chromosome X had the largest number of downregulated lncRNAs. Fifty-two differentially expressed lncRNAs could not be assigned to corresponding chromosomes.

Table 4. Top 20 significantly upregulated mRNAs in HPMECs treated with LPS. Fold: fold change. Chr: chromosome number. LPS 1 to 3 and Ctrl 1 to 3: normalized gene signals of each sample

Accession number	Gene symbol	P value	Fold	Chr	LPS1	LPS2	LPS3	Ctrl1	Ctrl2	Ctrl3
NM_000450	SELE	0.000051	86.46	chr1	10.77	10.79	10.97	4.37	4.13	4.72
NM_000584	IL8	0.000056	38.87	chr4	8.87	8.83	9.20	3.93	3.52	3.61
NM_173054	RELN	0.000061	19.37	chr7	9.97	9.97	10.16	5.88	5.55	5.83
NM_001078	VCAM1	0.000066	19.21	chr1	7.79	7.95	8.11	3.73	3.53	3.81
NM_006398	UBD	0.000071	17.98	chr6	8.08	7.86	7.95	3.62	3.86	3.90
NM_001511	CXCL1	0.000075	16.19	chr4	9.14	9.30	9.44	5.34	5.27	5.22
NM_177531	PKHD1L1	0.00008	16.40	chr8	7.12	7.34	7.57	3.26	3.27	3.40
NM_001130046	CCL20	0.000085	18.04	chr2	6.57	6.94	7.01	2.78	2.47	2.75
NM_001775	CD38	0.00009	15.38	chr4	7.71	7.90	8.18	3.97	4.02	3.97
NM_001565	CXCL10	0.000095	18.10	chr4	6.94	7.18	7.05	2.71	2.68	3.25
NM_001145938	MMP1	0.000114	17.17	chr11	10.53	10.56	10.66	6.79	6.09	6.56
NM_001128304	PLSCR4	0.000129	10.48	chr3	9.99	10.04	10.29	6.85	6.53	6.77
NM_000072	CD36	0.000134	7.82	chr7	5.58	5.84	5.93	2.84	2.77	2.83
NM_000201	ICAM1	0.000139	7.71	chr19	10.53	10.53	10.56	7.83	7.48	7.47
NM_001008	RPS4Y1	0.000148	7.20	chrY	6.59	6.92	6.87	3.88	3.98	3.96
NM_001006624	PDPN	0.000153	6.74	chr1	8.51	8.50	8.56	5.76	5.93	5.62
NM_014398	LAMP3	0.000158	5.78	chr3	7.90	7.91	8.09	5.47	5.40	5.44
NM_001781	CD69	0.000163	6.69	chr12	5.54	5.66	5.75	3.02	2.68	3.02
NM_001122665	DDX3Y	0.000168	6.86	chrY	6.25	6.39	6.75	3.67	3.61	3.77
NM_001712	CEACAM1	0.000173	5.97	chr19	8.24	8.21	8.23	5.58	5.88	5.48

Table 5. Top 20 significantly downregulated mRNAs in HPMECs treated with LPS. Fold: fold change. Chr: chromosome number. LPS 1 to 3 and Ctrl 1 to 3: normalized gene signals of each sample

Accession number	Gene symbol	P value	Fold	Chr	LPS1	LPS2	LPS3	Ctrl1	Ctrl2	Ctrl3
NM_001008540	CXCR4	0.000246	-4.21	chr2	7.27	7.16	7.20	9.30	9.09	9.46
NM_001130140	ERAP2	0.00046	-3.26	chr5	4.97	5.25	5.24	6.72	6.81	7.04
NM_018659	CYTL1	0.000528	-3.17	chr4	8.24	8.18	8.11	9.57	9.90	10.05
NM_019035	PCDH18	0.000586	-2.72	chr4	5.54	5.58	5.46	6.84	7.11	6.96
BC056414	PLVAP	0.000601	-2.58	chr19	6.94	6.84	6.89	8.18	8.39	8.19
NM_004476	FOLH1	0.000703	-2.37	chr11	4.45	4.31	4.42	5.61	5.55	5.76
NM_153696	FOLH1B	0.000708	-2.48	chr11	3.56	3.72	3.68	4.99	4.76	5.14
NM_006366	CAP2	0.000722	-2.31	chr6	5.75	5.64	5.87	6.97	6.91	7.00
NM_021194	SLC30A1	0.00079	-3.13	chr1	6.01	5.90	6.09	6.95	7.96	8.02
NM_001198	PRDM1	0.000839	-2.45	chr6	5.52	5.58	5.58	7.10	6.50	6.95
NM_001242628	GFOD1	0.000844	-2.08	chr6	6.19	6.16	6.09	7.15	7.25	7.20
NM_001199989	RASD1	0.000854	-2.21	chr17	4.92	4.85	4.93	5.85	6.09	6.19
NM_003654	CHST1	0.000888	-2.04	chr11	7.19	7.23	7.12	8.15	8.22	8.24
NM_001114403	UPK3BL	0.000897	-2.10	chr7	8.10	8.03	7.94	9.04	9.04	9.19
NM_006208	ENPP1	0.001009	-2.20	chr6	4.39	4.54	4.51	5.37	5.55	5.93
NM_004701	CCNB2	0.001019	-2.32	chr15	5.47	5.63	5.66	6.39	6.88	7.14
NM_021958	HLX	0.001063	-2.02	chr1	6.73	6.67	6.47	7.67	7.74	7.52
NM_003525	HIST1H2BI	0.001072	-2.41	chr6	5.44	5.33	5.52	6.26	6.64	7.19
NM_003537	HIST1H3B	0.001077	-2.46	chr6	5.79	5.75	5.83	6.58	7.06	7.61
NM_004460	FAP	0.001131	-2.01	chr2	3.88	3.85	4.04	4.88	4.79	5.12

GO and KEGG enrichment analyses

To explore the role of differentially expressed mRNAs in HPMECs treated with LPS, we performed GO and KEGG pathway enrichment analysis.

The results showed that upregulated genes were mainly associated with the following functions: response to lipopolysaccharide (ontology: biological process), integrin complex (ontology: cellular component), and cytokine activity (ontology: molecular function) (Fig. 2A). Downregulated genes were mainly associated with the following functions: male pronucleus (ontology: cellular component), C-terminal protein deglutamylation (ontology: biological process), and exopeptidase activity (ontology: molecular function) (Fig. 2B). The

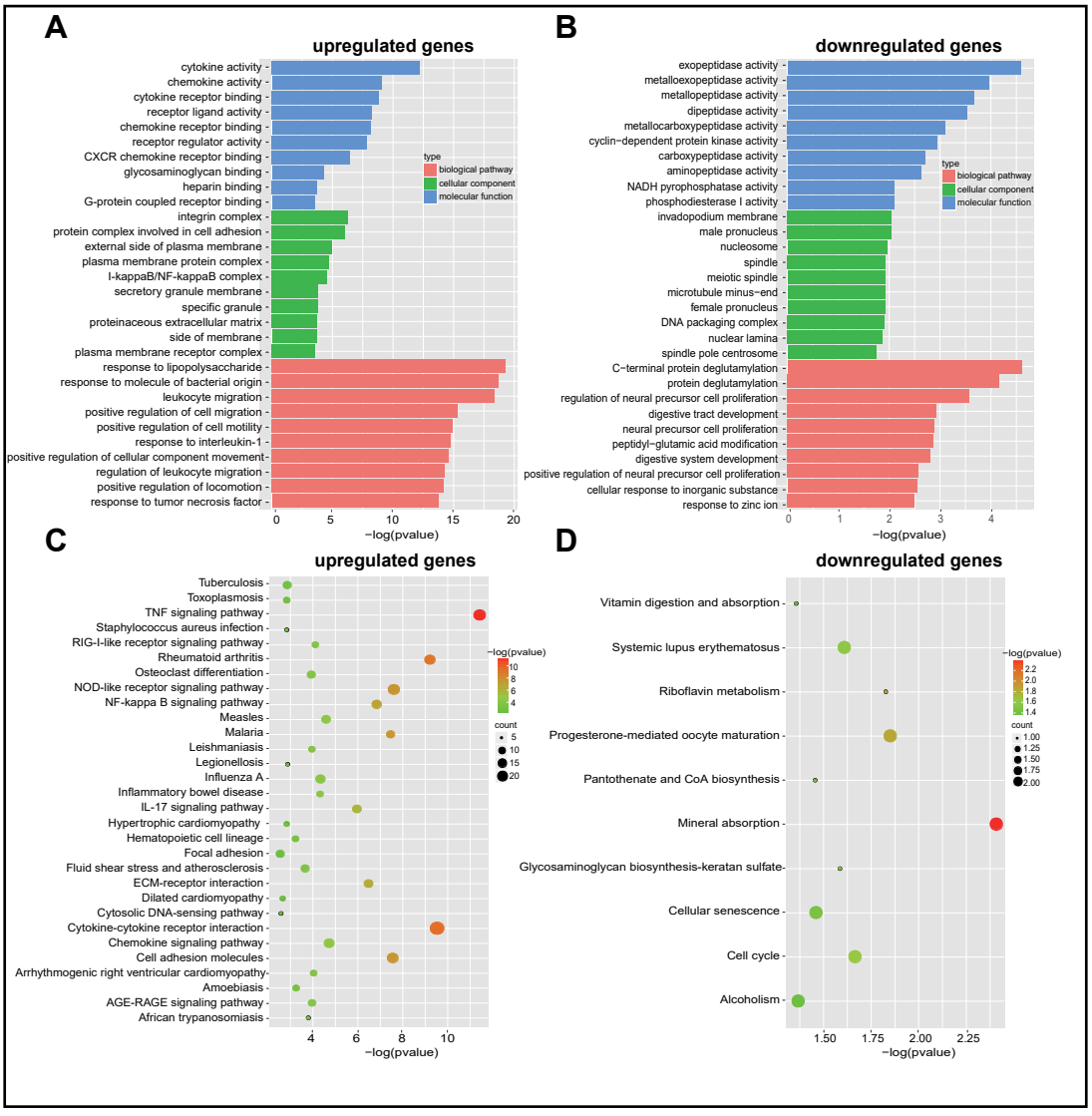

Fig. 2. GO and KEGG enrichment analyses for upregulated and downregulated mRNAs. GO enrichment analysis of upregulated (A) and downregulated (B) genes (Top 10, $p<0.05$). KEGG enrichment analysis for upregulated (C) and downregulated (D) genes ($p<0.05$). The red to green colors indicate high to low -log (p value) levels. Point size indicates the number of differentially expressed genes in the corresponding pathway.

results also indicated that upregulated genes were mainly associated with the following pathways: TNF signaling pathway, cytokine-cytokine receptor interaction, rheumatoid arthritis, NOD-like receptor signaling pathway, and cell adhesion molecules (CAMs) (Fig. 2C). The five most enriched pathways of downregulated genes were mineral absorption, progesterone-mediated oocyte maturation, riboflavin metabolism, cell cycle, and systemic lupus erythematosus (Fig. 2D).

These data suggested that upregulated mRNAs may directly participate in the process of pulmonary endothelial inflammation and barrier dysfunction.

LncRNA-mRNA coexpression networks with GO and KEGG enrichment analysis

To explore the potential biological functions of lncRNAs in HPMECs treated with LPS, we constructed a lncRNA-mRNA coexpression network based on 72 differentially expressed lncRNAs and 132 interacting differentially expressed mRNAs (Fig. 3A). Then, we performed GO and KEGG pathway enrichment analysis on the 132 mRNAs. We found that the most

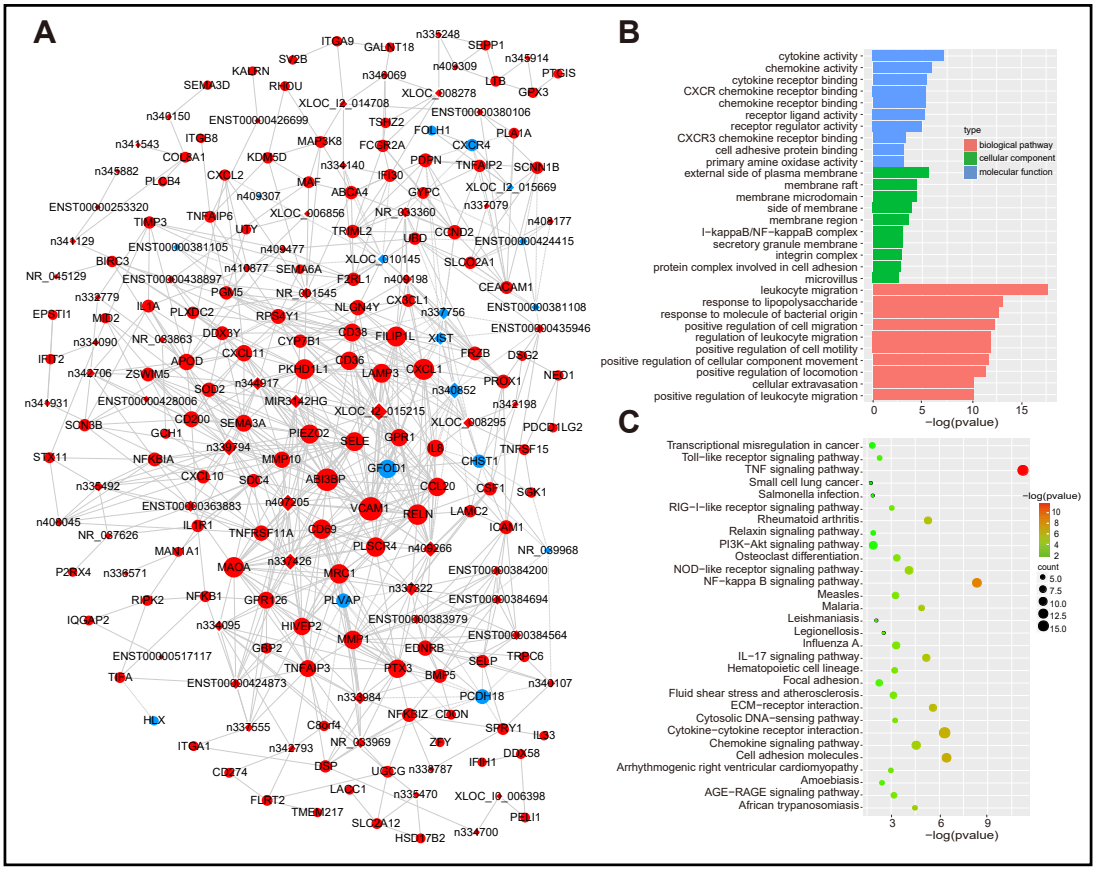

Fig. 3. Coexpression network of 72 differentially expressed lncRNAs and 132 interacting differentially expressed mRNAs with GO and KEGG analysis results. Coexpression network (A) of 72 lncRNAs and 132 interacting mRNAs. The diamonds represent lncRNAs, and the circles represent their correlated mRNAs. Blue dots and red dots indicate downregulated and upregulated lncRNAs and mRNAs. GO enrichment analysis (B) and KEGG enrichment analysis (C) of the 132 differentially expressed mRNAs ($p<0.05$).

enriched GOs were leukocyte migration (ontology: biological process), cytokine activity (ontology: molecular function), and external side of plasma membrane (ontology: cellular component) (Fig. 3B). The results also indicated that the 132 mRNAs were mainly associated with the following pathways: TNF signaling pathway, NF-κB signaling pathway, CAMs, cytokine-cytokine receptor interaction, and ECM-receptor interaction (Fig. 3C).

Real-time PCR validation of the microarray data

To validate the reliability of the microarray analysis results and to provide a research basis for further study, the expression levels of 6 lncRNAs (MIR3142HG, n344917, XLOC_l2_015215, n340107, n407205 and XIST) and 8 mRNAs (SELE, IL8, VCAM1, ICAM1, CXCL10, MMP10, ABI3BP and TRPC6) were determined by real-time PCR (Fig. 4). The results showed that MIR3142HG, XLOC_l2_015215, n340107 and n407205 were upregulated, whereas XIST was significantly downregulated. The expression of n344917 showed no significant change. SELE, IL8, VCAM1, ICAM1, CXCL10, MMP10, ABI3BP and TRPC6 were upregulated.

These data suggest that the expression levels of selected lncRNAs and mRNAs, except for n344917, detected by real-time PCR were similar to those detected by microarray analysis.

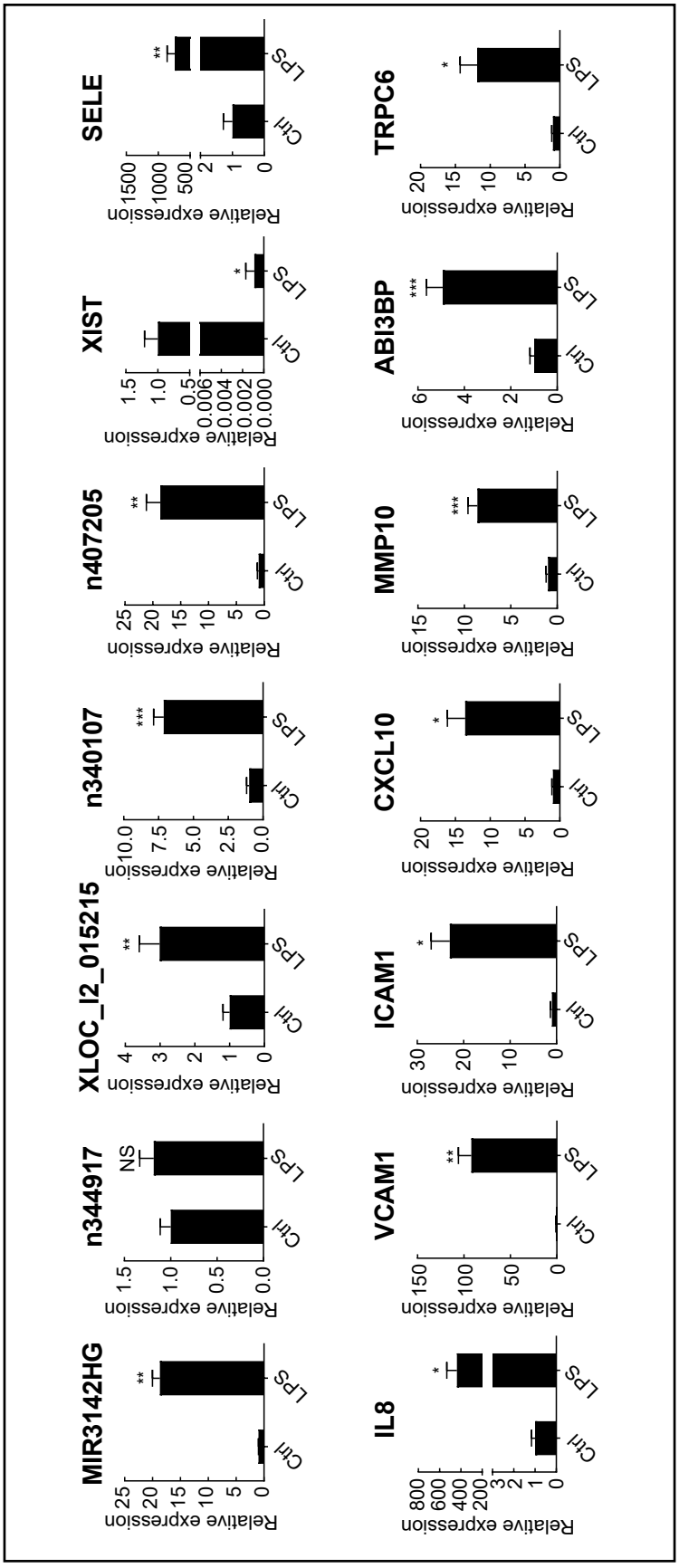

Fig. 4. Real-time PCR of six lncRNAs and eight mRNAs in HPMECs. All data are means ± SEM (n=3), Student's t-test. * P<0.05; ** P<0.01; *** P<0.001; NS, not significant.

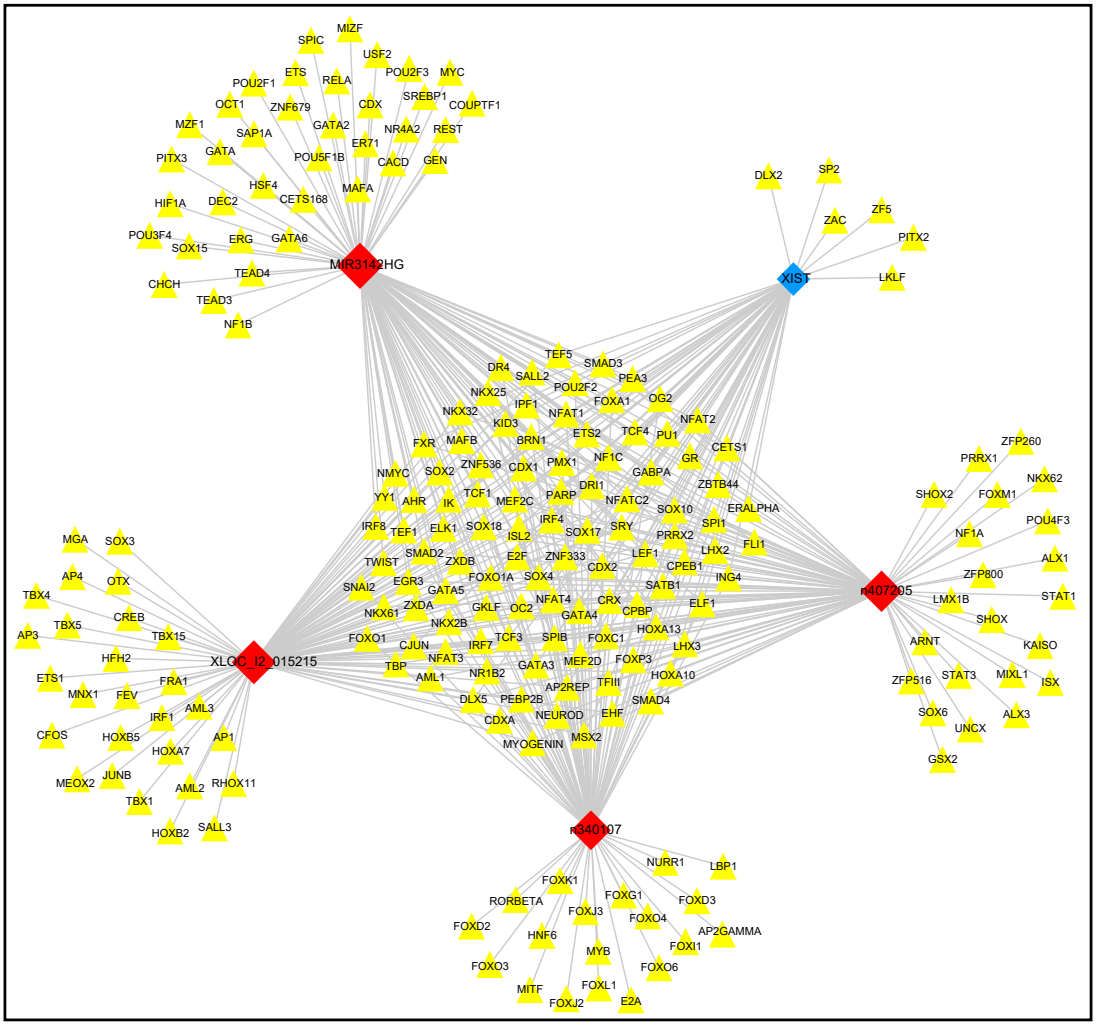

Fig. 5. TF regulatory network of the five validated lncRNAs. The diamonds represent lncRNAs. Blue and red dots indicate downregulated and upregulated lncRNAs. Yellowish triangles represent correlated TFs.

TF-lncRNA regulatory network

To understand the reason for the differential expression of the five validated lncRNAs (MIR3142HG, XLOC_l2_015215, n340107, n407205 and XIST), we predicted TFs mapping to these lncRNAs in the TRANSFACT professional database. The results showed that of the 214 TFs mapping to these lncRNAs (Fig. 5), 17 out of the 214 TFs were differently expressed (*fold change* > 1.2, $p < 0.05$). Seven differentially expressed TFs were predicted to regulate the transcription of XIST (Table 6), including 2 upregulated TFs (POU2F2 and BCL6) and 5 downregulated mRNAs (SOX18, MEF2C, SOX17, HOXB5 and ETS2). These findings indicate that differently expressed TFs might be one of the causes of the differential expression of lncRNAs.

Discussion

In this study, we established an experimental model of pulmonary endothelial inflammation and barrier dysfunction by stimulating HPMECs with LPS and investigated the expression profile of lncRNAs and mRNAs by microarray analysis. The results indicate that lncRNAs play an important role in LPS-induced pulmonary endothelial inflammation and barrier dysfunction.

Table 6. Differentially expressed TFs predicted to regulate the transcription of XIST. Range: the promoter region of XIST. Position: the position of the transcription start site. Sequence: the TF binding sequences in the promoter region

lncRNA	Range	Transcription factor	Relation	Position	sequence
XIST	chrX :-:73852253-73854253	SOX18	down	690	CAATGgg
XIST	chrX :-:73852253-73854253	SOX18	down	724	gaCATTG
XIST	chrX :-:73852253-73854253	SOX18	down	1536	CAATGgc
XIST	chrX :-:73852253-73854253	MEF2C	down	8	aaAAATA
XIST	chrX :-:73852253-73854253	MEF2C	down	94	aaAAATA
XIST	chrX :-:73852253-73854253	MEF2C	down	265	aaAAATA
XIST	chrX :-:73852253-73854253	MEF2C	down	317	aaAAATA
XIST	chrX :-:73852253-73854253	MEF2C	down	1665	aaAAATA
XIST	chrX :-:73852253-73854253	SOX17	down	597	gGACAA
XIST	chrX :-:73852253-73854253	HOXB5	down	743	aatAATTAat
XIST	chrX :-:73852253-73854253	ETS2	down	428	caGGAAG
XIST	chrX :-:73852253-73854253	POU2F2	up	1678	TATGCaaat
XIST	chrX :-:73852253-73854253	BCL6	up	391	ttctAGAAAg
XIST	chrX :-:73852253-73854253	BCL6	up	1565	ttctAGAAAg

An overwhelming majority of the transcriptions of the human genome are ncRNAs, which act as important transcriptional regulators in pathophysiologic processes [31-33]. In contrast to miRNAs, which regulate target genes by a posttranscriptional mechanism [34, 35], lncRNAs have the potential to regulate gene expression at posttranscriptional, transcriptional and epigenetic levels [36-39]. Furthermore, many lncRNAs have shown developmental stage-specific and tissue-specific expression patterns [40, 41].

The roles of ncRNAs in the pathogenesis of ALI and ARDS have attracted increasing attention. Recent studies have demonstrated that several miRNAs, such as miR-146, miR-155, and miR-221, serve as important regulators of inflammation-related mediators [33]. Hongbin Li et al. reported that the lncRNA CASC2 improved ALI by reducing lung epithelial cell apoptosis [42]. However, the function of lncRNA has not been investigated in pulmonary vascular injury associated with ALI and ARDS. Unlike two previous studies that analyzed the lncRNA expression profile in human umbilical vein endothelial cells [43] and human dermal microvascular endothelial cells [44] exposed to LPS, we identified the expression of LPS-responsive lncRNAs in HPMECs to investigate the role of lncRNAs in pulmonary endothelial injury.

In the present study, we determined the expression of 6 lncRNAs and 8 mRNAs by real-time PCR. The selection of these lncRNAs and mRNAs was based on the fold change and degree data in the lncRNA-mRNA coexpression network. Our results showed that the lncRNA and mRNA expression results determined by microarray analysis had good reliability and reproducibility. LPS successfully activated HPMECs because the expression of SELE, IL-8, VCAM-1, CCL20 and ICAM-1 increased significantly in the experimental model [45-47]. In addition, GO and KEGG pathway enrichment analyses also suggested that the differentially expressed mRNAs participated in pulmonary endothelial inflammation and barrier dysfunction. Notably, based on GO and KEGG pathway enrichment analyses of the lncRNA-mRNA coexpression network, we found that 72 differentially expressed lncRNAs might be mainly involved in the TNF signaling pathway, NF-κB signaling pathway, CAMs, cytokine-cytokine receptor interactions, and ECM-receptor interactions [48, 49]. Thus, we speculated that these lncRNAs might be associated with pulmonary endothelial inflammation and barrier dysfunction. Furthermore, we predicted differentially expressed TFs mapping to five selected lncRNAs that had been verified by real-time PCR with the help of the TRANSFAC professional database. The results suggested that differently expressed TFs might be one of the causes of the differential expression of lncRNAs.

The understanding of the diversity of gene regulation has greatly expanded in the past decade. There is increasing recognition that ncRNAs are important components of the gene regulatory network [50]. As the roles of lncRNAs become clearer, the knowledge acquired by this research will enable the understanding of how lncRNAs affect the initiation, progression, and resolution of pulmonary endothelial dysfunction associated with ALI and ARDS. Our data still need to be further validated in both vitro and vivo.

XIST, which is the master regulator of X chromosome inactivation, has been reported to play an important role in the pathogenesis of many diseases [51, 52]. H Yu et al. reported that XIST inhibition increased the blood-tumor barrier permeability in glioma endothelial cells [53]. Here, we found that XIST was significantly downregulated in HPMECs exposed to LPS by both microarray data and real-time PCR. This is the first study to determine the expression of XIST in HPMECs. The results may be helpful for further insights into the underlying role and mechanism of XIST in pulmonary endothelial inflammation and barrier dysfunction. The detailed function of XIST in pulmonary endothelial injury still needs to be further investigated.

Although not a genetic disease, ARDS has a certain hereditary susceptibility [54]. Three retrospective studies reported that men were at higher risk than women of incidence and mortality from ARDS [55-57]. In this study, we found that the distribution of differentially expressed lncRNAs was not equal. Interestingly, most differentially expressed lncRNAs on chromosome X were downregulated, while all differentially expressed lncRNAs on chromosome Y were upregulated. We speculate that the differential expression of lncRNAs on chromosomes X and Y may be one of the causes of sex-related differences in the morbidity and mortality of ARDS. Of course, this subject still requires more clinical research and further investigations.

Conclusion

In conclusion, we investigated the expression of lncRNAs and mRNAs in HPMECs treated with LPS by microarray analysis. We found that lncRNAs may be involved in LPS-induced pulmonary endothelial inflammation and barrier dysfunction. This is the first study to reveal the expression profile and potential role of lncRNAs in HPMECs. These findings provide a novel direction for both basic and clinical research for ALI and ARDS. Due to opportunities to identify novel therapeutic and preventive targets, our results may provide relevant information for future clinical interventions of ALI and ARDS.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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