Int J Clin Exp Pathol 2016;9(12):12275-12286
www.ijcep.com /ISSN:1936-2625/IJCEP0040321

Original Article

Long noncoding RNA expression profile of endothelial progenitor cells from deep vein thrombosis patients identified by microarray analysis

Ai-Min Qian*, Wen-Dong Li*, Ling-Shang Kong, Jian-Jie Rong, Feng-Rui Lei, Xiao-Long Du, Cheng-Long Li, Xiao-Qiang Li

*Department of Vascular Surgery, The Second Affiliated Hospital of Soochow University, Suzhou, China. *Equal contributors.*

Received September 20, 2016; Accepted September 27, 2016; Epub December 1, 2016; Published December 15, 2016

Abstract: The aim of this study was to identify differentially expressed lncRNAs in endothelial progenitor cells (EPCs) derived from the peripheral blood of deep vein thrombosis (DVT) patients compared with healthy controls. First, EPCs were obtained from DVT patients and healthy controls. Then, LncRNA gene expression profile analysis was performed by microarray. Bioinformatic analyses (gene ontology, pathway and network analysis) were applied. Finally, qRT-PCR analysis was performed to validate the results of microarray in EPCs derived from 21 DVT patients and nine healthy subjects. We found 275 lncRNA and 363 mRNAs that were abnormally expressed in EPCs derived from DVT patients (fold change ≥2.0, P<0.05 and FDR<0.05) with the genome-wide lncRNAs and mRNAs expression profile analysis. We also found that TCONS_00013536, ENST00000577218, ENST00000511042 and TCONS_00013917 were up-regulated, whereas ENST00000544704 was down-regulated. Expression of these five lncRNAs was significantly correlated to their nearby coding genes. Together, our results indicated that the lncRNA expression profile in EPCs from DVT patients was dramatically changed compared with healthy controls, and we identified a series of novel DVT-related lncRNAs which will provide important insights about the lncRNAs in DVT pathogenesis and may provide a new diagnosis way for DVT.

Keywords: lncRNAs, microarray, expression profiles, endothelial progenitor cell, deep vein thrombosis

Introduction

Deep venous thrombosis (DVT) is a common peripheral vascular disease with an incidence of about 1.0 person per 1,000 population per year [1], and a major cause of morbidity and mortality in various medical conditions. DVT may also lead to abnormal swelling and ulceration of lower limbs, post-thrombotic syndrome or even pulmonary embolism, which could result in an over 15% death rate in the first 3 months after diagnosis [2]. Anticoagulation therapy is currently the standard method for treatment of DVT. However, failure to remove existing thrombus and risk of pulmonary embolism hinder the use of the therapy [3]. Because of the lack of efficient therapy in DVT treatment, research to improve our understanding of the biology of DVT is urgently needed and novel strategies are required to identify targets for therapy and improve early detection of the disease.

Endothelial progenitor cells (EPCs) are derived from bone marrow and circulate in the peripheral blood, and have the capacity to differentiate into mature endothelial cells [4]. EPCs are considered as a biomarker of cardiovascular disease, as well as a potential regenerative medicine [5-7]. Previous studies have demonstrated that circulating EPCs have the potential to promote thrombus resolution as well as endothelial regeneration, revascularization and angiogenesis in animal models of intravenous thrombosis [4]. One study found that EPCs were increased at 24 h after DVT induction and peaked at 48 h thereafter [8]. Modarai et al. also found that EPCs were recruited into resolving venous thrombi and played a role in orchestrating thrombus recanalization [9]. In our previ-

Table 1. Clinical characteristics of the patients

	Patients			Normal			*P*-value
	DVT1	DVT2	DVT3	N1	N2	N3	
Age (years)	29	47	35	28	30	28	0.193
Gender	Male	Female	Female	Female	Male	Female	1.000
BMI	22	20	21	19	22	21	0.768
Thrombosis history	No	No	No	No	No	No	1.000
Immobility	No	No	Yes	No	No	No	1.000
Trauma	No	No	Yes	No	No	No	1.000

BMI: Body Mass Index.

Table 2. Primers used in qRT-PCR

Gene Name	Primer sequence	Tm (°C)	Product length (bp)
ENST00000544704	F: 5'CAAATCTGTGACAATGCCCC3' R: 5'GTTATGACTCAAGCGAAAATGG3'	60	72
TCONS_00013536	F: 5'TGAGCACACCATTGGAGAACCT3' R: 5'TTTGGGATTGGAGGGAGAGG3'	60	87
ENST00000577218	F: 5'CCGCCTCTAGTCCTCACAC3' R: 5'CGGAATGAATGGATGGTCT3'	60	235
ENST00000511042	F: 5'GCTGGATGTATGAACCCTGCT3' R: 5'TCCTGCTGGACTCTCGTGT3'	60	64
TCONS_00013917	F: 5'ATCCACCGAAGGTTTGAGG3' R: 5'GGGAGACGGGACTATATCCAG3'	60	98
GAPDH	F: 5'ACGGTGGTGGAGGAGCTCTT3' R: 5'GCCGGTTCAGGTACTCAGTCAT3'	60	157

F: Forward primer; R: Reverse primer.

ous studies, we also found that EPCs, as a promising therapeutic choice for DVT, played an important role in the process of venous thrombosis resolution [4, 10, 11]. This may open a new way for possible clinical translation and targeted cellular therapy for DVT [4].

Long noncoding RNAs (lncRNAs), a class of RNA molecules longer than 200 nucleotides, play critical roles in a series of biological processes, including genetic imprinting, immune response, tumorigenesis, cellular development and metabolism through comprehensive mechanisms [12-14]. Besides, LncRNAs also play critical roles in a variety of human diseases, such as cancer, neurodegeneration disease and cardiovascular disease [15-18]. Deregulation of lncRNAs has been demonstrated to be associated with angiogenesis, such as Tie1 antisense lncRNA, SENCR, and lncRNA MALAT1 [19-21]. Tie1 antisense lncRNA controls endothelial cell homeostasis and junction contacts *in vivo*, and was increased in patients with vascular anomalies, indicating therapeutic potential [19]. Despite these exciting developments, many more lncRNAs that play crucial roles in EPCs of DVT patients remain to be clarified.

LncRNAs expression profiles may help provide important insights into pathogenesis and a possible diagnostic strategy for DVT. Thus, in this study we examined lncRNAs and mRNAs that were differentially expressed in EPCs from DVT patients and healthy controls.

Materials and methods

Subjects

From January 2014 to January 2015, eighty milliliter of peripheral blood were collected from DVT patients (n = 3) and control subjects (n = 3) at the Second Affiliated Hospital of Soochow University, Suzhou, China. The DVT patients were confirmed by Color Doppler Ultrasound and lower extremity angiography and did not have a history of hypertension, diabetes mellitus and other chronic diseases. Patients and healthy controls were matched by age, gender and other risk factors (**Table 1**). The protocols were approved by the Institutional Review Board of the Second Affiliated Hospital of Soochow University and written informed consent was obtained from each participant.

Isolation and identification of EPCs

EPCs were isolated and characterized according to previous methods [22-24]. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Isopaque Plus (Histopaque-1077; Sigma, MO, USA) gradient centrifugation method. PBMCs were seeded onto a fibronectin-coated cell culture flask, cultured in endothelial basal medium-2 (EBM-2; Lonza, MD, USA) supplemented with 20% fetal bovine serum (FBS), vascular endothelial growth factor (VEGF; R&D

Systems, MN, USA), human recombinant long insulin-like growth factor-1 (R3-IGF), ascorbic acid, and hydrocortisone and maintained at 37°C, 5% CO_2. EPCs were characterized by confocal microscopy and flow cytometry. The cells were incubated with agglutinin 1 (FITC-UEA-1; Sigma Deisenhofen, Germany) and 1, 19-dioctadecyl-3, 3,3939-tetramethylindocar-bocyanine perchlorate (DiI)-labeled acetylated low density lipoprotein (DiI-Ac-LDL) as described previously [25]. Incorporation of DiI-Ac-LDL and binding of FITCUEA-1 were detected under a confocal microscope (Leica Microsystems GmbH, Germany). Cells with double positive staining of DiI-Ac-LDL and UEA-1 were identified as EPCs. EPCs were analyzed for surface expression of CD14, CD34, CD45, CD133 and VEGFR-2. Antibodies CD34, CD133 and VEGFR-2 were purchased from Miltenyi Biotec, Bergisch, Germany; CD14 and CD45 were purchased from BD Biosciences Pharmingen, CA, USA. The second passage of EPCs was used.

RNA extraction

Total RNA exaction was performed using TRIzol reagent (Life Technology, USA) according to the manufacturer's instructions. The quantity and quality of RNA were verified by a Nano Drop ND-1000 spectrophotometer. RNA integrity and genomic DNA contamination was examined by denatured agarose gel electrophoresis.

Microarray analysis and computational analysis

Sample preparation and microarray hybridization were performed by Kangchen Bio-tech, Shanghai P. R. China. The methods were similar to previous reports [14]. Briefly, RNA was purified from 1 μg total RNA after removal of rRNA (mRNA-ONLY Eukaryotic mRNA Isolation Kit, Epicentre). Each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias using a random priming method. The labeled cRNAs were hybridized onto the Human LncRNA Array v3.0 (Arraystar). After the slides were washed, the arrays were scanned by the Agilent Scanner G2505C. Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were carried out using the GeneSpring GX v12.1 software package (Agilent Technologies). Differentially expressed lncRNAs and mRNAs were identified through fold change filtering (Fold Change ≥2.0 or ≤0.5), paired t-test ($P<0.05$) and multiple hypothesis testing (FDR<0.05). *P* values and FDR were calculated by Microsoft Excel and MATLAB respectively. Pathway analysis and GO analysis were used to determine the roles of these differentially expressed mRNAs in these biological pathways or GO terms. R programme with Top GO package [26] was used to analyze the enrichment of differentially regulated mRNAs. We also used the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (http://www.genome.ad.jp/kegg/) to analyze the potential functions of these target genes in the pathways [27, 28].

Quantitative real-time polymerase chain reaction (qRT-PCR) validation and statistical analysis

To validate the results of microarray analysis, other EPCs samples from 21 DVT patients and nine control subjects were obtained. Total RNA was reverse-transcribed using I SuperScript™ III Reverse Transcriptase according to the manufacturer's protocol. Real-time PCR was performed with SYBR Premix Ex Taq (TaKaRa) on ABI7500 Real-time PCR System according to the manufacturer's protocol. PCR primers are listed in **Table 2**. The relative fold change was calculated using the $2^{-\Delta\Delta Ct}$ method normalized to GAPDH. Differences of lncRNAs between patients and normal were analyzed using paired t-test. A *P* value <0.05 was considered as statistically significant.

Results

EPCs characterization

First, EPCs were identified by morphology, fluorescence double-staining and flow cytometry. The flow cytometry analysis results (**Figure 1A**) matched with the previously described EPC phenotype [23, 29]. Most adherent cells were double stained by DiI-AcLDL and FITC-UEA-I (**Figure 1B**). These results were consistent with the characterization of late-outgrowth EPCs.

Expression profile of lncRNAs and mRNAs in EPCs from DVT patients

To identify differentially expressed lncRNAs and mRNAs in EPCs from DVT patients com-

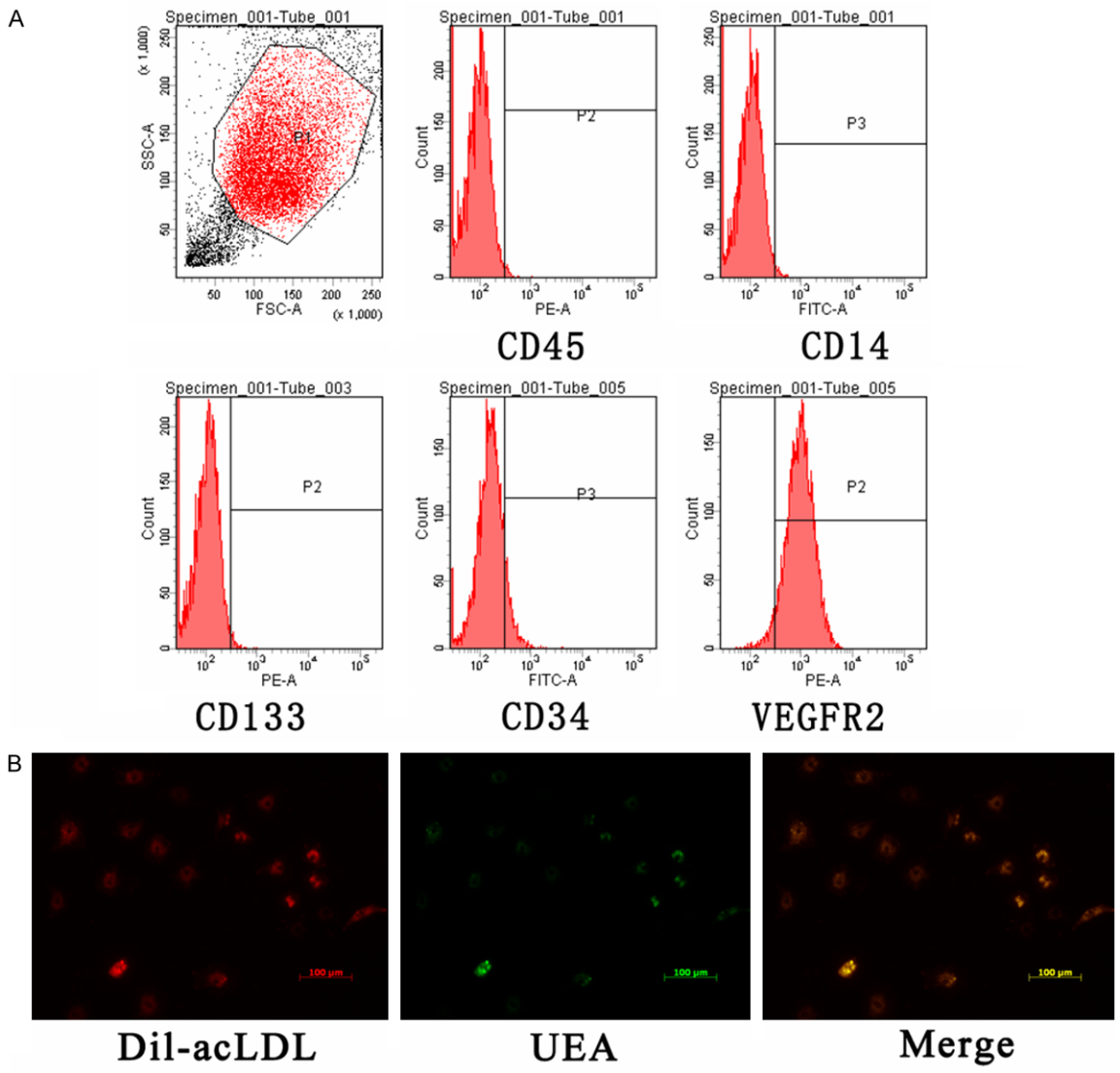

Figure 1. Characterization of EPCs. A: Flow cytometry analysis of EPCs. Cells were labeled with antibodies to CD45, CD14, CD133, CD34 and VEGFR2. Histograms represent cell number (Y-axis) versus fluorescence intensity (X-axis, log scale). Cells (10,000) were acquired and gates were set on living cells. B: DiI-Ac-LDL uptake and FITC-UEA-I binding of isolated EPCs were determined by fluorescence microscopy.

pared with controls, we performed microarray analysis and identified 30,586 differentially expressed lncRNAs and 26,109 mRNAs. After expression level normalization, we calculated the fold-change (DVT vs. control) and *p* value. A total of 275 lncRNAs and 363 mRNAs were confirmed as significantly differentially expressed in EPCs from DVT patients compared with EPCs from control subjects (fold change ≥2.0 or ≤0.5, P<0.05 and FDR<0.05). Among these, 83 lncRNAs and 127 mRNAs were consistently up-regulated in all DVT groups, and 192 lncRNAs and 236 mRNAs were consistently down-regulated. The number of deregulated lncRNAs and mRNAs varied in different patients. The differentially expression of these lncRNAs suggest that they may play important roles in the regulation of the function of EPCs during the development of DVT.

We used hierarchical clustering analysis to arrange samples into groups based on their expression levels, which allowed us to hypothesize on the relationships among samples (**Figure 2**). The resulting dendrogram shows the relationships between the lncRNA and mRNA expression patterns between samples.

LncRNA classification and subgroup analysis

LncRNAs are classified into different subgroups, such as antisense lncRNAs, lncRNAs

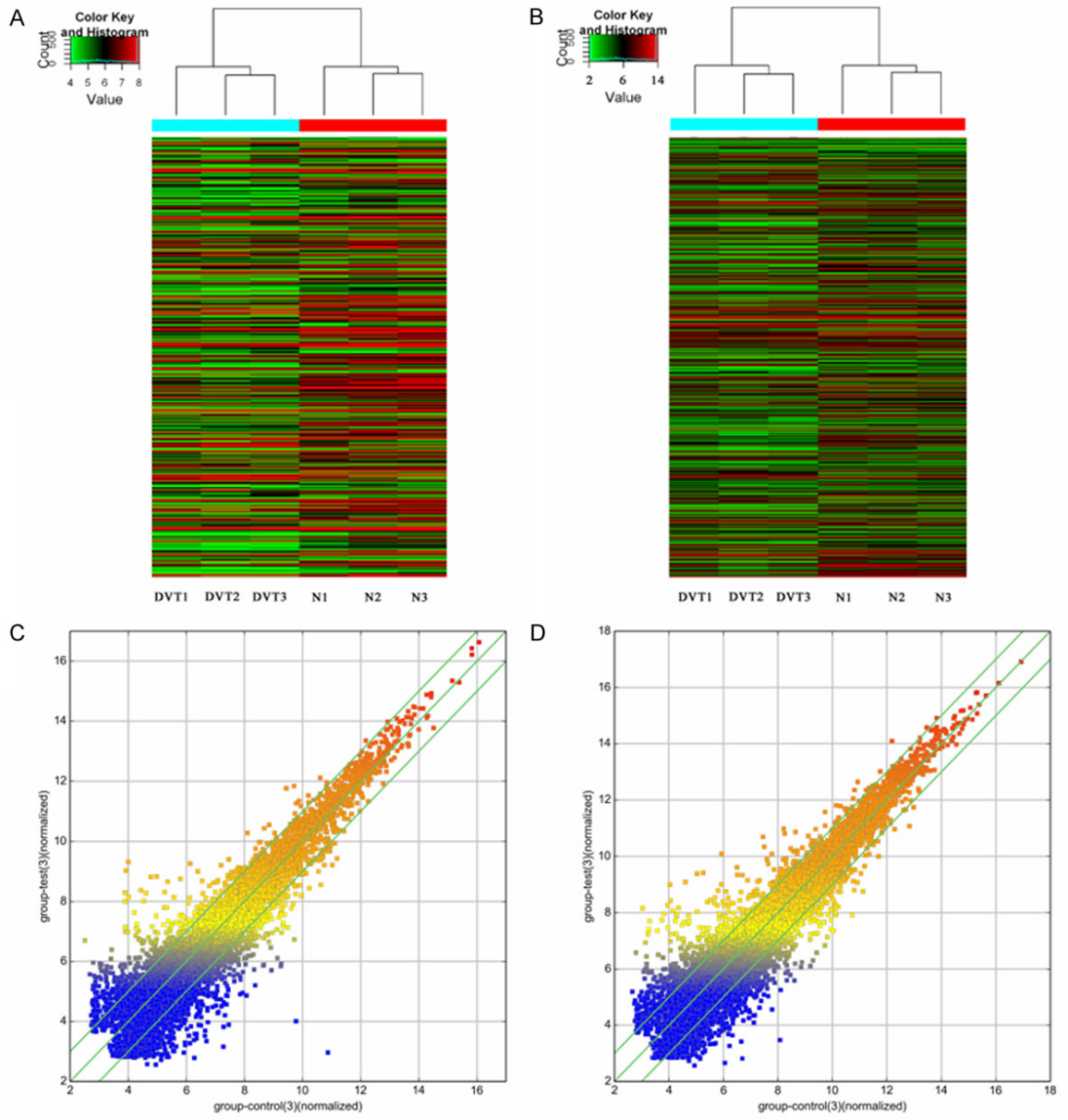

Figure 2. Comparison of the expression profiles of lncRNAs and mRNAs in EPCs between the DVT group and the paired healthy control group. (A) Differentially expressed lncRNAs and (B) differentially expressed mRNAs were analyzed using hierarchical clustering. "Red" indicates high relative expression, and "green" indicates low relative expression. (C, D) The scatter plot is a visualization method used for assessing the lncRNA (C) and mRNA (D) expression variations between DVT and control samples. The values of the X and Y axes in the scatter plot are the averaged normalized signal values of the group (log2 scale). The green lines are 2 fold change lines.

with enhancer-like functions and large intergenic noncoding RNAs [14]. To further investigate potential functions of these DVT-associated lncRNAs, lncRNA classification and subgroup analysis was performed.

Natural antisense lncRNA transcripts are RNA molecules that are transcribed from the opposite DNA strand and overlap in part with sense RNA, and can exert regulatory effects on the sense RNA through epigenetic mechanisms. The profiling data indicated that five such lncRNAs were dysregulated in DVT samples.

Using the GENCODE annotation [30] of the human genome, Orom et al. defined a set of lncRNAs with enhancer-like functions in human

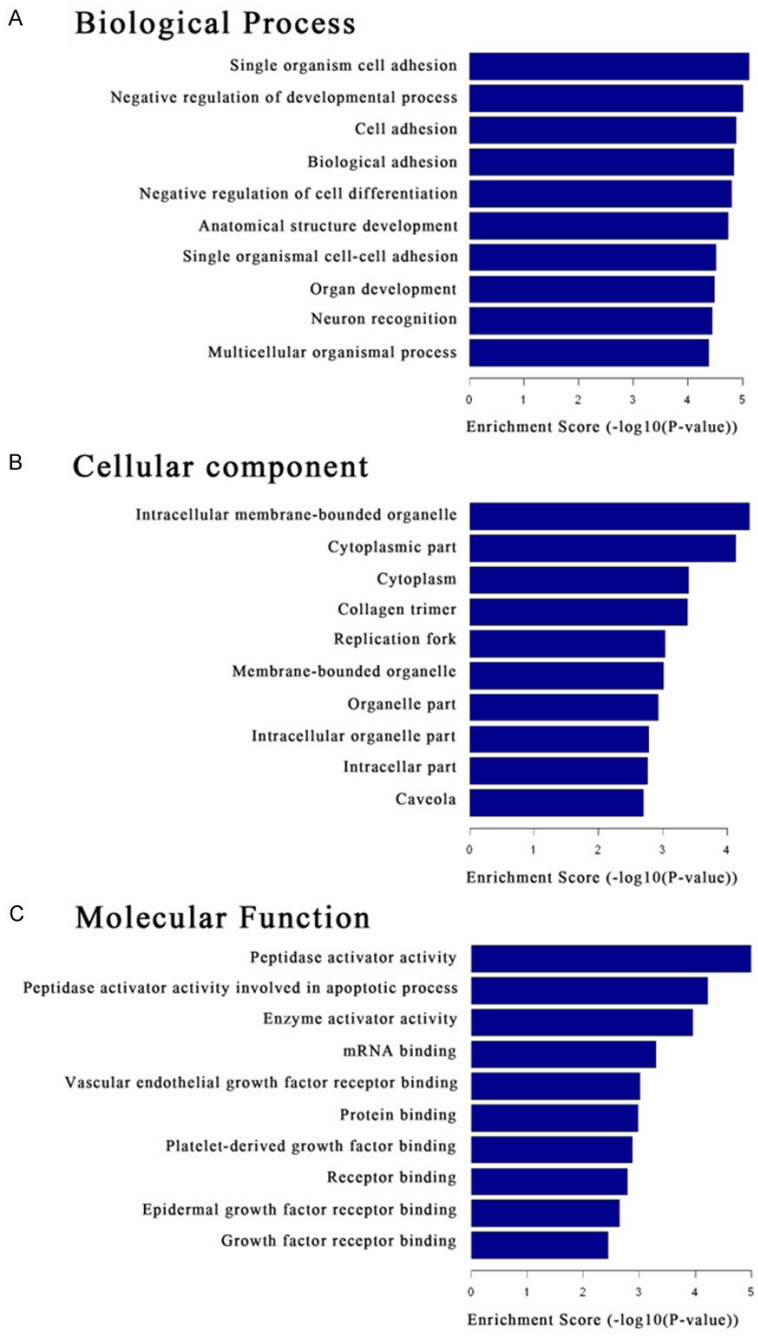

Figure 3. Gene ontology (GO) enrichment analysis for differentially expressed mRNAs. A: GO analysis of mRNAs according to biological process. B: GO analysis of mRNAs according to cellular component. C: GO analysis of mRNAs according to molecular function.

cell lines [31]. The profiling data showed that four such lncRNAs were differentially expressed in our DVT samples.

According to previous report [32], we also selected lncRNAs and nearby mRNAs to perform the analyses. The profiling data indicated that five such lncRNAs were dysregulated in EPCs from DVT samples, and these were located in nearby aberrantly expressed coding genes.

Bioinformatic analysis of mRNA expression in DVT

Our results identified a total of 363 mRNAs that were differentially expressed in DVT patients. Using R programming and top GO package [26], we analyzed the enrichment of these 363 differentially regulated mRNAs. The most significant functional groups consisted of cell adhesion, cell differentiation and the regulation of developmental processes (**Figure 3**). The most significant molecular function included enzyme activator activity, VEGF receptor binding, platelet-derived growth factor binding and other growth factor receptor binding. These changes may contribute to EPCs homing and migrating into thrombi and differentiating into mature endothelial cells.

Pathway analysis

Significant pathways of differential genes were compared with the KEGG database to further identify target mRNAs among the 3,278 identified genes. Through the pathway analysis, we identified 14 pathways that were significantly enriched among the upregulated transcripts (**Table 3**) and 30 pathways (**Table 4**) that were significantly enriched among the downregulated transcripts. Among them, leukocyte transendothelial migration and cytosolic DNA-sensing pathway were the most enriched networks (**Figure 4**). Of note, pathways associated with migration may play important roles in EPCs homing to thrombi.

Table 3. The 14 pathways that corresponded to up-regulated transcripts

Pathway ID	Definition	Fisher-*P* value	FDR	Enrichment score
hsa04670	Leukocyte transendothelial migration-Homo sapiens (human)	0.004721208	0.4491231	2.325947
hsa00512	Mucin type O-Glycan biosynthesis-Homo sapiens (human)	0.007848341	0.4491231	2.105222
hsa05160	Hepatitis C-Homo sapiens (human)	0.00838558	0.4491231	2.076467
hsa05162	Measles-Homo sapiens (human)	0.008686624	0.4491231	2.061149
hsa05164	Influenza A-Homo sapiens (human)	0.009043315	0.4491231	2.043672
hsa05202	Transcriptional misregulation in cancer-Homo sapiens (human)	0.009589817	0.4491231	2.01819
hsa05168	Herpes simplex infection-Homo sapiens (human)	0.01169454	0.4694521	1.932017
hsa05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)-Homo sapiens (human)	0.01668933	0.5862126	1.777561
hsa00983	Drug metabolism-other enzymes-Homo sapiens (human)	0.02297849	0.7174396	1.638678
hsa04640	Hematopoietic cell lineage-Homo sapiens (human)	0.02941917	0.7687683	1.53137
hsa04380	Osteoclast differentiation-Homo sapiens (human)	0.03009413	0.7687683	1.521518
hsa04514	Cell adhesion molecules (CAMs)-Homo sapiens (human)	0.04366333	0.9110974	1.359883
hsa05161	Hepatitis B-Homo sapiens (human)	0.04475318	0.9110974	1.349176
hsa05416	Viral myocarditis-Homo sapiens (human)	0.04539275	0.9110974	1.343013

Table 4. The 30 pathways that corresponded to down-regulated transcripts

Pathway ID	Definition	Fisher *P* value	FDR	Enrichment score
hsa04623	Cytosolic DNA-sensing pathway-Homo sapiens (human)	0.00263103	0.3459388	2.579874
hsa03440	Homologous recombination-Homo sapiens (human)	0.003006624	0.3459388	2.521921
hsa04961	Endocrine and other factor-regulated calcium reabsorption-Homo sapiens (human)	0.003782637	0.3459388	2.422205
hsa04978	Mineral absorption-Homo sapiens (human)	0.004924395	0.3459388	2.307647
hsa00240	Pyrimidine metabolism-Homo sapiens (human)	0.007990745	0.3916327	2.097413
hsa04640	Hematopoietic cell lineage-Homo sapiens (human)	0.01240606	0.3916327	1.906366
hsa05215	Prostate cancer-Homo sapiens (human)	0.01306934	0.3916327	1.883746
hsa03430	Mismatch repair-Homo sapiens (human)	0.01323153	0.3916327	1.87839
hsa04510	Focal adhesion-Homo sapiens (human)	0.01472141	0.3916327	1.832051
hsa04919	Thyroid hormone signaling pathway-Homo sapiens (human)	0.01528542	0.3916327	1.815723
hsa04720	Long-term potentiation-Homo sapiens (human)	0.01533082	0.3916327	1.814435
hsa05034	Alcoholism-Homo sapiens (human)	0.0170253	0.3986758	1.768905
hsa05218	Melanoma-Homo sapiens (human)	0.01928739	0.4169044	1.714726
hsa04916	Melanogenesis-Homo sapiens (human)	0.02305891	0.4552372	1.637161
hsa00230	Purine metabolism-Homo sapiens (human)	0.03373287	0.4552372	1.471947
hsa04621	NOD-like receptor signaling pathway-Homo sapiens (human)	0.03566406	0.4552372	1.447769
hsa05221	Acute myeloid leukemia-Homo sapiens (human)	0.03566406	0.4552372	1.447769
hsa04725	Cholinergic synapse-Homo sapiens (human)	0.03723153	0.4552372	1.429089
hsa04726	Serotonergic synapse-Homo sapiens (human)	0.03861753	0.4552372	1.413216
hsa04151	PI3K-Akt signaling pathway-Homo sapiens (human)	0.0394896	0.4552372	1.403517
hsa04512	ECM-receptor interaction-Homo sapiens (human)	0.03986393	0.4552372	1.39942
hsa00533	Glycosaminoglycan biosynthesis-keratan sulfate-Homo sapiens (human)	0.04152981	0.4552372	1.38164
hsa04012	ErbB signaling pathway-Homo sapiens (human)	0.04157717	0.4552372	1.381145
hsa04730	Long-term depression-Homo sapiens (human)	0.04189233	0.4552372	1.377865
hsa04974	Protein digestion and absorption -Homo sapiens (human)	0.04333446	0.4552372	1.363167
hsa03030	DNA replication-Homo sapiens (human)	0.04344184	0.4552372	1.362092
hsa04540	Gap junction-Homo sapiens (human)	0.04513596	0.4552372	1.345477
hsa04810	Regulation of actin cytoskeleton-Homo sapiens (human)	0.04601429	0.4552372	1.337107
hsa05414	Dilated cardiomyopathy-Homo sapiens (human)	0.04698177	0.4552372	1.328071
hsa05219	Bladder cancer-Homo sapiens (human)	0.04974438	0.4605383	1.303256

Genomic location of differentially expressed lncRNAs

Several studies have suggested that lncRNAs can regulate high order chromosomal dynamics, subcellular structural organization and telomere biology [33]. LncRNAs originate from complex loci that include lncRNAs and associated protein-coding genes, and a number of lncRNAs have been reported to regulate the

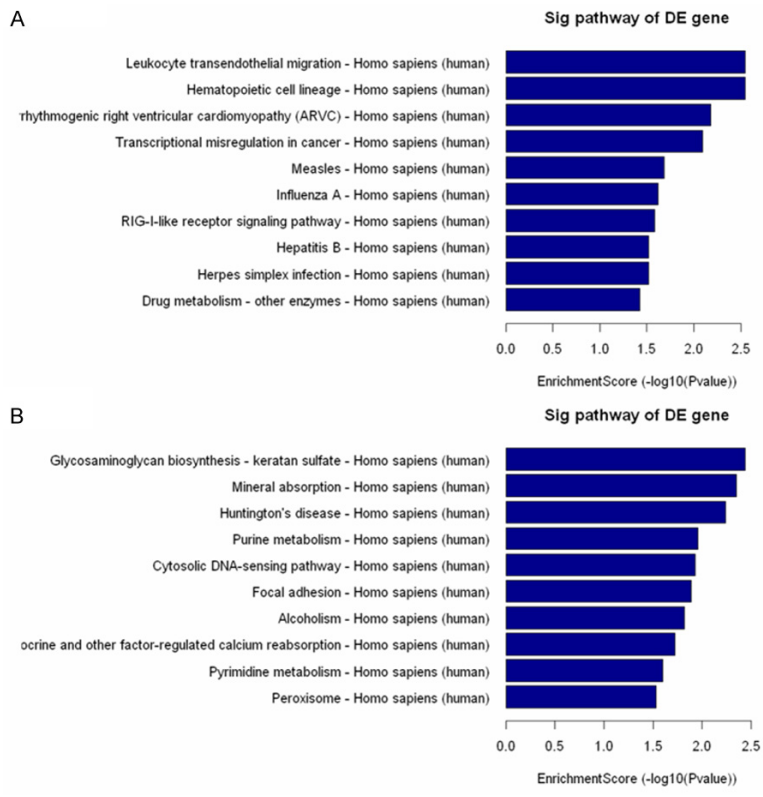

Figure 4. Pathway analysis. The top 10 pathways of the coding genes associated with upregulated lncRNAs (A) and downregulated lncRNAs (B) are listed.

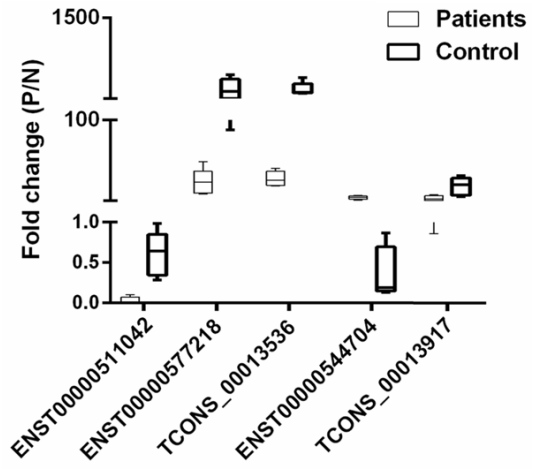

Figure 5. Quantitative RT-PCR validation of five differentially expressed lncRNAs in DVT samples. TCONS_00013536, ENST00000577218, ENST00000511042 and TCONS_00013917 were upregulated, whereas ENST00000544704 was downregulated in five pairs of DVT samples compared with control samples.

expression of adjacent protein-coding genes [34, 35]. The relationships of lncRNAs and nearby coding genes identified here include bidirectional, exon sense overlapping, intergenic, intro sense overlapping, intronic antisense and natural antisense. The 275 differentially expressed lncRNAs in DVT samples included 24 directional sequences, 29 exon sense-overlapping sequences, 123 intergenic sequences, 12 intro sense-overlapping sequences, 42 intronic antisense and 37 natural antisense.

Five dysregulated lncRNAs in DVT samples

To verify the microarray data, we randomly selected five differentially expressed lncRNAs including four upregulated lncRNAs and one downregulated lncRNA and validated their expression levels by quantitative RT-PCR (qRT-PCR) in sets of EPCs samples from 21 DVT patients compared with nine control subjects. The expression pattern results of the five lncRNAs were consistent with the microarray data. TCONS_00013536, ENST00000577218, ENST00000511042 and TCONS_00013917 were upregulated, whereas ENST00000544704 was downregulated in EPCs from DVT samples compared with controls (**Figure 5**). Additionally, microarray data also provided the nearby coding genes of these differentially expressed lncRNAs. We found that the changes of lncRNAs and their nearby coding genes are basically identical to the results of qRT-PCR analysis (**Figure 6**). These results, which were consistent with the previous reports [14], provided strong evidence that lncRNAs had intrinsic cis-regulatory capacity to their own locus.

Discussion

During the past decades, the roles of EPCs in the prognosis of thrombosis have been extensively studied [4, 8-11]. However, the molecular mechanisms of EPCs in the resolution of thrombosis have not yet been fully elucidated.

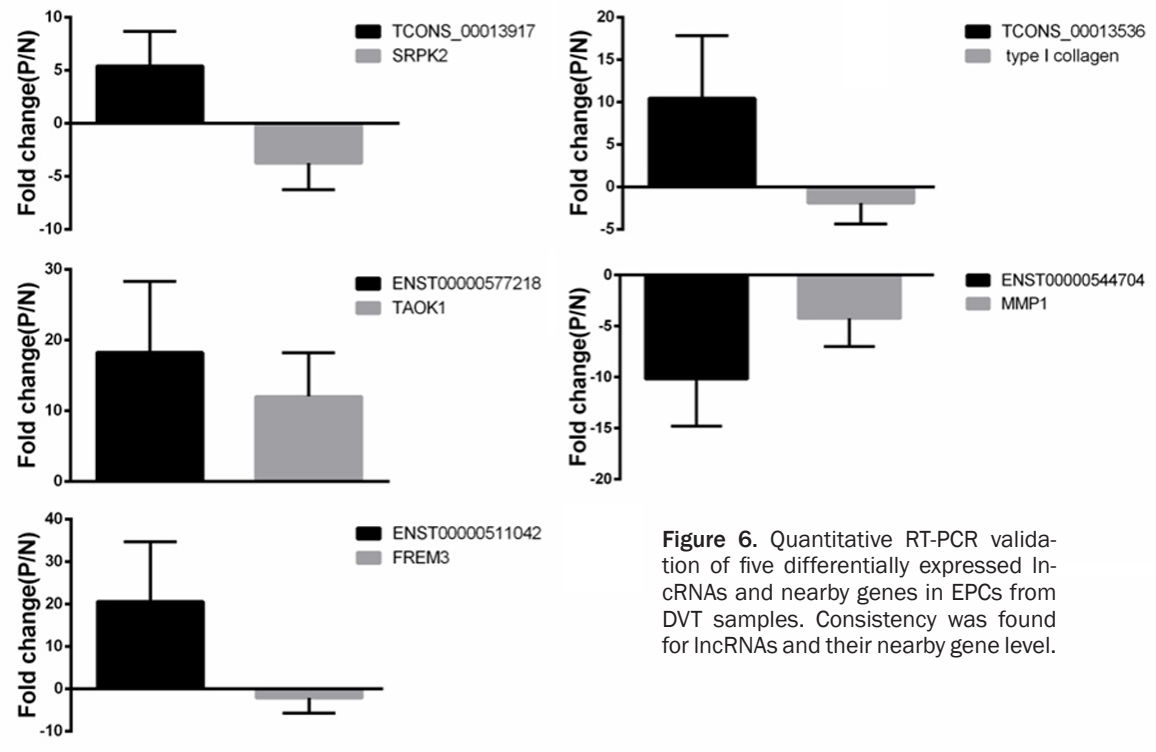

Figure 6. Quantitative RT-PCR validation of five differentially expressed lncRNAs and nearby genes in EPCs from DVT samples. Consistency was found for lncRNAs and their nearby gene level.

MicroRNAs (miRNAs) are a class of noncoding small RNAs that exert a critical role in the function regulation of EPCs. Altered expression of miRNAs in EPCs has been shown to be involved in thrombi resolution [10, 11]. However, the roles of lncRNAs in EPCs and in thrombi formation and resolution have not yet been determined. Although the majority of lncRNA could be functional, only few have been demonstrated to be involved in biological processes, such as regulation of basal transcription machinery functions, RNA splicing and translation, and epigenetic regulation [36-38]. Moreover, the exact roles of lncRNA in cells are less clear compared with those of miRNAs. Therefore, we conducted the present study to obtain insight into the roles of lncRNA in EPCs and a role in pathogenesis of DVT.

Recent evidences have shown that lncRNAs play an important role in gene expression regulation through cis (i.e., on neighboring genes) or trans (distant genes) manners [39]. However, prediction of the specifics is relative difficulty based on lncRNA sequence [40, 41]. LncRNAs have been implicated in multiple functions, particularly in vascular diseases such as in atherosclerosis [42] and varicose vein disease [43]. Previous studies have shown expression of the hypoxia-inducible factor 1α (HIF1α) gene, which is commonly involved in angiogenic events [44, 45], is regulated through lncRNA-p21 [46]. Thus, the aberrant expression of at least one lncRNA has been linked to vascular events.

To date, there have been no reports of differentially expressed lncRNAs in DVT samples. Our data is the first to show a total of 275 differentially expressed lncRNAs and 363 mRNAs in DVT samples, with a fold change of 2 or more. GO and pathway analyses predicted that downregulated and upregulated transcripts of mRNAs were associated with cellular process (ontology: biological process), cell (ontology: cellular component), and binding (ontology: molecular function). We found that these genes were mainly involved in cell adhesion and cell differentiation, which may suggest that the differently expressed lncRNAs could regulate migration and differentiation via influencing the expression of these genes. To further understand the functions of lncRNAs, we used pathway analysis to associate these differentially expressed lncRNAs with their target genes and found 44 pathways that corresponded to transcripts; the most enriched network was the cytosolic DNA-sensing pathway, which was composed of 64 targeted genes. One of these

pathways, the gene category 'leukocyte transendothelial migration and focal adhesion pathways', has been reported to be involved in the homing of EPCs [4].

Here we demonstrated that TCONS_00013536, ENST00000577218, ENST00000511042 and TCONS_00013917 were upregulated and ENST00000544704 was downregulated in EPCs from DVT samples compared with controls. Furthermore, expressions of five lncRNAs were significantly correlated with their nearby coding genes, which have not been described before. For example, the downregulated lncRNA ENST000000544704 in DVT was found to be located near the gene encoding MMP1. MMP-1 functions to promote proliferation and migration of vascular cells (vascular smooth muscle cells and endothelial cells) [47, 48], vessel remodeling, angiogenesis and tumor progression [49]. A natural antisense association between the downregulated lncRNA ENST00000544704 and MMP1 gene may help illuminate how lncRNAs regulate gene expression in vessel components. The upregulated lncRNA ENST00000511042 FREM3 is important in the regulation of in the structure and function of basement membrane [50] and therefore may be an important regulator in angiogenesis.

The current study of lncRNAs in EPCs from DVT samples is a proof-of-principle that lncRNAs have a probable role in vein thrombosis pathogenesis. Further studies should be made to fully understand this disease with the aims of developing effective treatment strategies. Our present study on the potential relationships between lncRNAs and EPCs in DVT presents a novel area for further investigations into the targeted lncRNAs and the potential therapeutic strategies for this disease.

Acknowledgements

This work was supported by grants from the Scientific and Technological Research Projects of Jiangsu Province (No. BL2014043), Graduate Research and Innovation Program in Colleges and Universities of Jiangsu Province (No. KYLX15_1202).

Disclosure of conflict of interest

None.

Address correspondence to: Xiao-Qiang Li, Department of Vascular Surgery, The Second Affiliated Hospital of Soochow University, 1055 Sanxiang Rd, Suzhou 215000, Jiangsu, China. E-mail: vasculars@126.com; flytsg@126.com

References

[1] Saez-Gimenez B, Berastegui C, Loor K, Lopez-Meseguer M, Monforte V, Bravo C, Santamaría A, Roman A. Deep vein thrombosis and pulmonary embolism after solid organ transplantation: an unresolved problem. Transplant Rev (Orlando) 2015; 29: 85-92.

[2] Piazza G, Goldhaber SZ. Acute pulmonary embolism: part I: epidemiology and diagnosis. Circulation 2006; 114: e28-32.

[3] Weitz JI, Eikelboom JW, Samama MM. New antithrombotic drugs: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. Chest 2012; 141: e120S-151S.

[4] Li WD, Li XQ. Endothelial progenitor cells accelerate the resolution of deep vein thrombosis. Vascul Pharmacol 2016; 83: 10-16.

[5] Grisar JC, Haddad F, Gomari FA, Wu JC. Endothelial progenitor cells in cardiovascular disease and chronic inflammation: from biomarker to therapeutic agent. Biomark Med 2011; 5: 731-744.

[6] Bakogiannis C, Tousoulis D, Androulakis E, Briasoulis A, Papageorgiou N, Vogiatzi G, Kampoli AM, Charakida M, Siasos G, Latsios G, Antoniades C, Stefanadis C. Circulating endothelial progenitor cells as biomarkers for prediction of cardiovascular outcomes. Curr Med Chem 2012; 19: 2597-2604.

[7] Smadja DM, Duong-van-Huyen JP, Dal Cortivo L, Blanchard A, Bruneval P, Emmerich J, Gaussem P. Early endothelial progenitor cells in bone marrow are a biomarker of cell therapy success in patients with critical limb ischemia. Cytotherapy 2012; 14: 232-239.

[8] Alessio AM, Beltrame MP, Nascimento MC, Vicente CP, de Godoy JA, Silva JC, Bittar LF, Lorand-Metze I, de Paula EV, Annichino-Bizzacchi JM. Circulating progenitor and mature endothelial cells in deep vein thrombosis. Int J Med Sci 2013; 10: 1746-1754.

[9] Modarai B, Burnand KG, Sawyer B, Smith A. Endothelial progenitor cells are recruited into resolving venous thrombi. Circulation 2005; 111: 2645-2653.

[10] Meng Q, Wang W, Yu X, Li W, Kong L, Qian A, Li C, Li X. Upregulation of MicroRNA-126 Contributes to Endothelial Progenitor Cell Function in Deep Vein Thrombosis via Its

Target PIK3R2. J Cell Biochem 2015; 116: 1613-1623.
[11] Wang W, Li C, Li W, Kong L, Qian A, Hu N, Meng Q, Li X. MiR-150 enhances the motility of EPCs in vitro and promotes EPCs homing and thrombus resolving in vivo. Thromb Res 2014; 133: 590-598.
[12] Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. Cell 2009; 136: 629-641.
[13] Wang KC, Chang HY. Molecular mechanisms of long noncoding RNAs. Mol Cell 2011; 43: 904-914.
[14] Zhu J, Liu S, Ye F, Shen Y, Tie Y, Zhu J, Jin Y, Zheng X, Wu Y, Fu H. The long noncoding RNA expression profile of hepatocellular carcinoma identified by microarray analysis. PLoS One 2014; 9: e101707.
[15] Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, Hung T, Argani P, Rinn JL, Wang Y, Brzoska P, Kong B, Li R, West RB, van de Vijver MJ, Sukumar S, Chang HY. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 2010; 464: 1071-1076.
[16] Johnson R. Long non-coding RNAs in Huntington's disease neurodegeneration. Neurobiol Dis 2012; 46: 245-254.
[17] Faghihi MA, Modarresi F, Khalil AM, Wood DE, Sahagan BG, Morgan TE, Finch CE, St Laurent G 3rd, Kenny PJ, Wahlestedt C. Expression of a noncoding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of beta-secretase. Nat Med 2008; 14: 723-730.
[18] Fiedler J, Breckwoldt K, Remmele CW, Hartmann D, Dittrich M, Pfanne A, Just A, Xiao K, Kunz M, Müller T, Hansen A, Geffers R, Dandekar T, Eschenhagen T, Thum T. Development of Long Noncoding RNA-Based Strategies to Modulate Tissue Vascularization. J Am Coll Cardiol 2015; 66: 2005-2015.
[19] Li K, Blum Y, Verma A, Liu Z, Pramanik K, Leigh NR, Chun CZ, Samant GV, Zhao B, Garnaas MK, Horswill MA, Stanhope SA, North PE, Miao RQ, Wilkinson GA, Affolter M, Ramchandran R. A noncoding antisense RNA in tie-1 locus regulates tie-1 function in vivo. Blood 2010; 115: 133-139.
[20] Bell RD, Long X, Lin M, Bergmann JH, Nanda V, Cowan SL, Zhou Q, Han Y, Spector DL, Zheng D, Miano JM. Identification and initial functional characterization of a human vascular cell-enriched long noncoding RNA. Arterioscler Thromb Vasc Biol 2014; 34: 1249-1259.
[21] Yan B, Yao J, Liu JY, Li XM, Wang XQ, Li YJ, Tao ZF, Song YC, Chen Q, Jiang Q. lncRNA-MIAT regulates microvascular dysfunction by functioning as a competing endogenous RNA. Circ Res 2015; 116: 1143-1156.
[22] Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 2003; 348: 593-600.
[23] Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, Li T, Isner JM, Asahara T. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci U S A 2000; 97: 3422-3427.
[24] Kong L, Hu N, Du X, Wang W, Chen H, Li W, Wei S, Zhuang H, Li X, Li C. Upregulation of miR-483-3p contributes to endothelial progenitor cells dysfunction in deep vein thrombosis patients via SRF. J Transl Med 2016; 14: 23.
[25] Ma FX, Zhou B, Chen Z, Ren Q, Lu SH, Sawamura T, Han ZC. Oxidized low density lipoprotein impairs endothelial progenitor cells by regulation of endothelial nitric oxide synthase. J Lipid Res 2006; 47: 1227-1237.
[26] Alexa A, Rahnenfuhrer J. topGO: Enrichment analysis for Gene Ontology. R Package Version 2010; 2230.
[27] Kanehisa M, Goto S, Kawashima S, Okuno Y, Hattori M. The KEGG resource for deciphering the genome. Nucleic Acids Res 2004; 32: D277-280.
[28] Zhang C, Han L, Zhang A, Yang W, Zhou X, Pu P, Du Y, Zeng H, Kang C. Global changes of mRNA expression reveals an increased activity of the interferon-induced signal transducer and activator of transcription (STAT) pathway by repression of miR-221/222 in glioblastoma U251 cells. Int J Oncol 2010; 36: 1503-1512.
[29] Rehman J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 2003; 107: 1164-1169.
[30] Harrow J, Denoeud F, Frankish A, Reymond A, Chen CK, Chrast J, Lagarde J, Gilbert JG, Storey R, Swarbreck D, Rossier C, Ucla C, Hubbard T, Antonarakis SE, Guigo R. GENCODE: producing a reference annotation for ENCODE. Genome Biol 2006; 7 Suppl 1: S4 1-9.
[31] Orom UA, Derrien T, Beringer M, Gumireddy K, Gardini A, Bussotti G, Lai F, Zytnicki M, Notredame C, Huang Q, Guigo R, Shiekhattar R. Long noncoding RNAs with enhancer-like function in human cells. Cell 2010; 143: 46-58.
[32] Cabili MN, Trapnell C, Goff L, Koziol M, Tazon-Vega B, Regev A, Rinn JL. Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. Genes Dev 2011; 25: 1915-1927.
[33] Amaral PP, Mattick JS. Noncoding RNA in development. Mamm Genome 2008; 19: 454-492.

[34] Martianov I, Ramadass A, Serra Barros A, Chow N, Akoulitchev A. Repression of the human dihydrofolate reductase gene by a non-coding interfering transcript. Nature 2007; 445: 666-670.
[35] Feng J, Bi C, Clark BS, Mady R, Shah P, Kohtz JD. The Evf-2 noncoding RNA is transcribed from the Dlx-5/6 ultraconserved region and functions as a Dlx-2 transcriptional coactivator. Genes Dev 2006; 20: 1470-1484.
[36] Dinger ME, Amaral PP, Mercer TR, Mattick JS. Pervasive transcription of the eukaryotic genome: functional indices and conceptual implications. Brief Funct Genomic Proteomic 2009; 8: 407-423.
[37] Mercer TR, Dinger ME, Mattick JS. Long noncoding RNAs: insights into functions. Nat Rev Genet 2009; 10: 155-159.
[38] Dong R, Jia D, Xue P, Cui X, Li K, Zheng S, He X, Dong K. Genome-wide analysis of long noncoding RNA (lncRNA) expression in hepatoblastoma tissues. PLoS One 2014; 9: e85599.
[39] Khachane AN, Harrison PM. Mining mammalian transcript data for functional long non-coding RNAs. PLoS One 2010; 5: e10316.
[40] Hung T, Chang HY. Long noncoding RNA in genome regulation: prospects and mechanisms. RNA Biol 2010; 7: 582-585.
[41] Qureshi IA, Mattick JS, Mehler MF. Long noncoding RNAs in nervous system function and disease. Brain Res 2010; 1338: 20-35.
[42] Hu YW, Zhao JY, Li SF, Huang JL, Qiu YR, Ma X, Wu SG, Chen ZP, Hu YR, Yang JY, Wang YC, Gao JJ, Sha YH, Zheng L, Wang Q. RP5-833A20.1/miR-382-5p/NFIA-dependent signal transduction pathway contributes to the regulation of cholesterol homeostasis and inflammatory reaction. Arterioscler Thromb Vasc Biol 2015; 35: 87-101.
[43] Li X, Jiang XY, Ge J, Wang J, Chen GJ, Xu L, Xie DY, Yuan TY, Zhang DS, Zhang H, Chen YH. Aberrantly expressed lncRNAs in primary varicose great saphenous veins. PLoS One 2014; 9: e86156.
[44] Kumarswamy R, Bauters C, Volkmann I, Maury F, Fetisch J, Holzmann A, Lemesle G, de Groote P, Pinet F, Thum T. Circulating long noncoding RNA, LIPCAR, predicts survival in patients with heart failure. Circ Res 2014; 114: 1569-1575.
[45] Vausort M, Wagner DR, Devaux Y. Long noncoding RNAs in patients with acute myocardial infarction. Circ Res 2014; 115: 668-677.
[46] Yang F, Zhang H, Mei Y, Wu M. Reciprocal regulation of HIF-1alpha and lincRNA-p21 modulates the Warburg effect. Mol Cell 2014; 53: 88-100.
[47] Shi ZD, Ji XY, Qazi H, Tarbell JM. Interstitial flow promotes vascular fibroblast, myofibroblast, and smooth muscle cell motility in 3-D collagen I via upregulation of MMP-1. Am J Physiol Heart Circ Physiol 2009; 297: H1225-1234.
[48] Blackburn JS, Brinckerhoff CE. Matrix metalloproteinase-1 and thrombin differentially activate gene expression in endothelial cells via PAR-1 and promote angiogenesis. Am J Pathol 2008; 173: 1736-1746.
[49] Mazor R, Alsaigh T, Shaked H, Altshuler AE, Pocock ES, Kistler EB, Karin M, Schmid-Schönbein GW. Matrix metalloproteinase-1-mediated up-regulation of vascular endothelial growth factor-2 in endothelial cells. J Biol Chem 2013; 288: 598-607.
[50] Pavlakis E, Chiotaki R, Chalepakis G. The role of Fras1/Frem proteins in the structure and function of basement membrane. Int J Biochem Cell Biol 2011; 43: 487-495.