

LncRNA LYPLAL1-DT in Type 2 Diabetes With Macrovascular Complication Contributes Protective Effects on Endothelial Cells via Regulating the miR-204-5p/SIRT1 Axis

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Abstract

Background: Long noncoding RNAs (lncRNAs) are involved in diabetes related diseases. However, the role of lncRNAs in the pathogenesis of type 2 diabetes with macrovascular complication (DMC) has seldomly been recognized. This study aimed to screen lncRNA profiles of leukocytes from DMC patients in order to explore the protective role of lncRNA LYPLAL1-DT in endothelial cells (EC) under high glucose (HG) and inflammatory conditions (IS).

Methods: RNA sequencing was performed for critically pair-grouped blood samples of DMC patients and healthy control. Then the differentially expressed (DE) lncRNAs from circulating leukocytes were identified. Real-time PCR analyses were used to select the DE-lncRNAs within expanding cohorts. CCK8, transwell, Western blot, dual-luciferase system, and RIP were used to investigate the influence and molecular mechanisms of validated DE-lncRNAs in EC under HG and IS conditions. RNA sequencing was also used to identify DE-lncRNAs in exosomes isolated from the DMC serum and healthy control.

Results: A total of 477 DE-lncRNAs were identified between DMC and healthy control. The enrichment and pathway analysis showed that most of them belonged to inflammatory, metabolic, and vascular diseases. A set of 12 of the 16 lncRNAs was validated as significant DE-lncRNAs in expanding cohorts. Furthermore, these DE-lncRNAs were shown to be significantly related to hypoxia, high glucose, and TNF- α stimulus (IS) in EC with an apparent metabolic memory of high glucose, especially novel lncRNA LYPLAL1-DT. LYPLAL1-DT overexpression results in the promotion of proliferation, migration of EC, as well as an elevation of autophagy under HG, and IS conditions. Overexpressed LYPLAL1-DT reduces the adhesion of monocytes to EC, boosts anti-inflammation, and suppresses inflammatory molecules secreted in the medium. Mechanistically, LYPLAL1-DT acts as ceRNA by downregulating miR-204-5p, therefore enhancing SIRT1 and protecting EC autophagy function; thus, alleviating apoptosis. Finally, exosome sequencing revealed LYPLAL1-DT expression was 4 times lower in DMC cells than in healthy samples.

Conclusion: We identified 12 DE-lncRNAs related to DMC. Out of the 12 DE-lncRNAs lncRNA LYPLAL1-DT was identified to have protective effects on EC as ceRNA mediated through the miR-204-5p/SIRT1 pathway. Therefore, it inhibits the autophagy of EC as well as modulating systemic inflammation. This approach could be regarded as a new potential therapeutic target in DMC.

Introduction

Over the past three decades, the prevalence of diabetes has sharply increased worldwide. It is a major cause of blindness, kidney failure, heart attacks, stroke, and lower limb amputation [1]. Diabetes is usually recognized as a vascular disease characterized by vasoregulatory changes, inflammatory activation resulting from high glucose (HG), and hypoxia (HO). Diabetic vascular complications include micro and macrovascular dysfunctions occurring in several organs, such as the muscles, eyes, skin, kidneys, brain, and heart [2]. More than 50 % of diabetic patients die of diabetic macrovascular

complications (DMC) such as cardiovascular disease, making it a major cause of morbidity and mortality. The leading cause of DMC is hyperglycemia induced endothelial injury or dysfunction, and inflammation, therefore resulting in atherosclerosis [3]. Hence, understanding the molecular mechanisms causing endothelial injury under hyper-glucose and inflammation would be helpful as a DMC therapeutic agent.

Long non-coding RNAs (lncRNAs) are recognized as a class of noncoding single-stranded RNAs longer than 200 bases with no evident function of coding proteins [4]. Most lncRNAs are located in the nucleus, or both the nucleus and the cellular plasma where they function as molecular scaffolds, motifs in alternative splicing, or in the modification of chromatin structures [5]. Growing evidence has proved that dysregulated or dysfunctional lncRNAs have emerging roles in more than 200 diseases, and new associations continue to accumulate in diabetic literature [6]. lncRNAs may operate through quite a few molecular mechanisms, as an example lncRNA MALAT1 either acts as a competing endogenous RNA (ceRNA) for miR-205-5p in human mesenchymal stem cells regulating VEGF production and improving endothelial cell tube formation [7], or upregulate the inflammatory mediators IL-6 and TNF- α through activation of serum amyloid antigen 3 [8]. Studies investigating the link between lncRNAs and the development of diabetic complications have just begun [4]. Advances in sequencing and microarray technology enable the identification of large numbers of putative lncRNA loci and accelerate the progress in this research field [9, 10]. Although several studies have focused on the correlation of lncRNA and vascular diseases [11-13], the role and characteristics of vascular abnormality in diabetes caused by lncRNAs involved in hyperglucose and inflammation remain poorly understood [11, 14-17]. In the present study, we screened differentially expressed lncRNAs in circulating leukocytes isolated from diabetic macrovascular complicated individuals using RNA sequencing, we identified a few new lncRNAs related to DMC abnormal endothelial cell and further explored the protective effects of lncRNA LYPLAL1-DT as a ceRNA to inhibit miR-204-5p and upregulate SIRT1 in order to promote autophagy of endothelial cell (EC), as well as ameliorate inflammation of EC. These results reveal a novel lncRNA as a potential diagnostic marker and therapeutic target for DMC treatment in a clinical practice.

Methods

Subjects

Human blood samples collected from the Department of Endocrinology, Chinese PLA General Hospital, were grouped into either a discovery cohort with healthy control (CTR, n=6), type 2 diabetes mellitus with macrovascular complication (DMC, n=6), or a validation cohort with healthy control (CTR, n=36), type 2 diabetes mellitus with macrovascular complication (DMC, n=46) by clinic examination. All consenting adult subjects (18-65 years old) with no past medical history were consecutively enrolled between July 2016 and April 2018. The whole blood samples were subsequently collected separately in tubes containing RNA after overnight fasting for 10 to 12 h. The subjects in the healthy control group were: 1) healthy with a negative diagnosis of DMC as defined by the World Health Organization (WHO) and with normal blood biochemical indexes; 2) free from all endocrine disease, and 3) aged 18-65 years old

without a gender bias. Exclusion criteria were similar to our previous study [18]. The detailed grouped information of all patients was shown in Table 1. The study was approved by the Ethics Committee of Chinese PLA General Hospital (Permitted No. S2016-147-03) and all patients were given informed consent.

Blood samples collection, exosome extraction, RNA sequencing and analysis

The whole blood sample was collected after fasting 10-14h, and total RNA was extracted from peripheral leukocytes as previously reported [18]. Exosomes derived from DMC patients' serum were extracted via PEG precipitate method by VEXTM Exosome Isolation Reagent (Vazyme, Nanjing, China). The RNA sequencing process, investigation flow, and data analysis design and methods were similar to our previous work and shown in Figure 1A. RNA sequencing was performed by Annoroad Gene Tech. Co., Ltd.

Cell culture and high glucose, hyperoxia, and inflammatory stimulus

Endothelial cell (EC) line human umbilical vein endothelial cells (HUVECs) were purchased from the Shanghai Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI 1640 medium (Corning) at 37 °C with 5% CO₂, and supplemented with 10% fetal bovine serum (Vistech). Cells were cultured with a media consisting of 25/30 mM D-glucose (high glucose, HG) or 5 mM D-glucose and 20mM mannitol (Equilibrium osmotic pressure) (normal glucose, NG). Hypoxia treatment was proceeded by culture cell under 37 °C with 5% CO₂. The inflammatory stimulus was performed by adding TNF- α at a concentration of 100ng/mL. For metabolism memory detection, the cells were treated with 25 mM glucose for 24h first, then cultured in normal medium with a glucose concentration for 72h. At the end of treatment, the expression level of lncRNAs was determined.

RNA extraction and Real-time PCR

For further validation of lncRNA expression from RNA-sequencing results, a total of 16 lncRNA were measured by real-time PCR in independent expanding cohorts of healthy control (n=36), and DMC (n=46) groups. The full primer list is presented in Supplementary Table S1. Total RNA extraction and PCR were performed as followed in our previous report [18]. The 2- $\Delta\Delta$ CT method was used to quantify the relative expression of each lncRNA, using β -actin as an internal control. The relative expression of each examined gene was determined in triplicates. Differences in the lncRNA expressions among the groups were evaluated with a one-way analysis of variance (ANOVA) using SPSS 18.0 software. A significant difference was considered to be $P < 0.05$.

RNA samples from cultured endothelial cells were extracted using RNA isolation Reagent (Vazyme, Nanjing, China). Complementary DNA (cDNA) from 2 μ g total RNA was synthesized using 5X All-In-One RT MasterMix (ABM, Zhenjiang, China). The amplification reaction volume was 20 μ L and contained 10 μ L EvaGreen 2X qPCR MasterMix (ABM, Zhenjiang, China), 1 μ L cDNA, 2 μ L amplification primers, and 7 μ L ddH₂O.

Real-time PCR measured the expression levels of the predicted miRNAs and targeted genes in EC. A similar process was used for the peripheral leukocytes. The cDNA of the miRNA was synthesized using a miRNA 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China), and real-time PCR was performed using miRNA Universal SYBR qPCR MasterMix (Vazyme, Nanjing, China). The primers for each gene are also listed in Supplementary Table S2.

Construction of lncRNA LYPLAL1-DT overexpression in an EC line

The overexpression of LYPLAL1-DT in HUVECs and the corresponding control cells (OC) were constructed using a pLenti-GIII-CMV-CBH-GFP-2A-Puro vector (ABM, Zhenjiang, China). Cells were transduced for 24 h with a recombinant lentivirus and cultured for 72 h. The transduction efficacy was verified by GFP expression and detected by Real-time PCR.

Cell transfection

Cells were transfected with miR-204-5p mimic and a negative control (HANBIO, Shanghai, China). MiR-204-5p mimic was transfected at a final concentration of 50nM via an RNAfit reagent (HANBIO, Shanghai, China), following the manufacturer's instruction.

Cell viability, transwell assay, and Western blot

The viability and migration of HUVEC was assessed using CCK8 (Vazyme, Nanjing, China), MTT (Vazyme, Nanjing, China), a transwell assay, and a Western blot as performed in our previous studies. [19]. The primary antibodies used for Western blot analysis were LC3(CST), Caspase 3 (CST), β -actin (CST), SIRT1 (CST), Alix (CST), and TSG101 (CST).

Luciferase assay

To explore the role of the competing endogenous RNA LYPLAL1-DT in ECs, we cloned the 3'-untranslated regions (UTR) of LYPLAL1-DT downstream of the Renilla luciferase gene to generate RLuc- LYPLAL1-DT wild type vector (WT) and the corresponding mutant type vector (MUT); thus using the Firefly luciferase gene as an internal reference. For the luciferase reporter assays, HUVECs were plated in a 24-well culture plates, and then transfected with either the WT or the MUT construct with and without miRNA mimic or negative control mimic. Luciferase activities were measured using a Dual Luciferase Reporter Assay System (Vazyme, Nanjing, China), and every transfected well was analyzed in triplicate. The RLuc-SIRT1 wild type vector (WT) was generated to detect relative luciferase expression, following the above experimental steps, in order to discover the relations between miR-204-5p and SIRT1.

HUVEC-Monocyte adhesion and cytokines detection

To detect monocyte-endothelial interactions, in vitro static cell adhesion assays were performed. Human monocytic THP-1 cells were labeled via a fluorescent dye DiI (YEASEN, Shanghai, China), then were co-cultured with HG-treated and TNF- α -stimulated HUVEC in a 6-well plate for 30 mins. After washing with

PBS three times, fluorescence (red) of the adherent THP-1 cells were quantified under a fluorescence microscope.

Cytokines in the cell culture medium were analyzed using ProcartaPlex Multiplexing Immunoassay kit (eBioscience, USA) on luminex 200 (Luminex, USA). Results were calculated using the ProcartaPlex Analyst 1.0 software.

Apoptosis Detection and RNA Immunoprecipitation Assay (RIP)

The apoptosis level of HG-treated and TNF- α -stimulated HUVEC cells was detected by a TUNEL BrightGreen Apoptosis Detection Kit (Vazyme, Nanjing, China).

RIP was conducted using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore). Briefly, HUVECs were lysed and incubated with magnetic beads conjugated with AGO2 antibodies. After washing with a wash buffer, immunoprecipitated RNA was detected by real-time PCR.

Statistical analysis

Unless otherwise stated, all experiments were performed at a minimum of triplicates. Statistical analysis was performed using SPSS 19.0 software (SPSS Inc., USA). The data is represented as the mean \pm SEM. Comparisons between groups were made using a student's t-test or one-way ANOVA. Statistically significant differences were set as a P value <0.05 .

Results

Transcriptomic profiles in patients with Diabetic macrovascular complications

To determine the transcriptomic profiles, the transcriptome sequencing in the discovery cohorts composed of 6 patients diagnosed with DMC and 6 health controls. The data process flow chart is shown in Fig. 1a. The information of the DMC participants in both the discovery and the expanded cohorts are shown in Table 1, and demonstrates that most clinical characteristics were not significantly different between the two cohorts. Thus, the lncRNAs selected in the discovery cohort are reliably validated in the expanded cohort.

First, we described the transcriptomic profiles of DMC to identify the critical genes and lncRNAs in the DMC patients. A total of 14,978 lncRNAs and 17,172 mRNAs were detected in leukocytes from DMC patients. Of these, 9,235 lncRNAs have been registered in databases and defined as known. Out of the 9,235 lncRNA's, 4,647 were upregulated, and 4,588 were downregulated. The other 5,743 lncRNAs were identified for the first time and were defined as either upregulated (3,379) or downregulated (2,364) novel lncRNAs. After optimization using an adjusted p -value (threshold of <0.05) and change fold >2 , we identified 477 significantly differentially expressed (DE) lncRNAs (DMC-lncRNAs) (Table S3), among which 245 were downregulated and 232 were upregulated (Fig. 1b). The top 20 DMC-lncRNAs are shown in Table 2. We further analyzed the different biotypes of 477 DE-lncRNAs. The results indicated that 172

lncRNAs (36.1%) were antisense, 253 (53.0%) were intergenic (lincRNA), and 52 (10.9%) belonged to other types (sense-overlapping or sense-intronic) (Fig. 1c, Table S3). Further, 798 DMC-mRNAs were found by similar optimization (Table S4), with 491 downregulated and 307 upregulated (Fig. S1a). Both DMC-lncRNA and DMC-mRNA were distinguishable within DMC patients and the healthy controls by hierarchical clustering (Fig. 1d, Fig. S1b) and principal component analysis (Fig. 1e, Fig. S1c). This data clearly illustrates a distinguishable differential expression profile of the leukocytes between DMC patients and the healthy controls.

Features of DE-lncRNAs and mRNAs identified in patients with DMC

The Gene Ontology (GO) enrichment analysis was applied to classify DMC patients' DE-lncRNAs and DE-mRNAs. Under the biological process category, metabolic process, single organism process, response to stimulus, and cellular process were the top 4 DE-lncRNAs; similar items were found for the DE-mRNAs (Fig. 2a, Fig. S2). Under the molecular function category, both DE-lncRNAs and DE-mRNAs showed the highest percentages in catalytic activity and signal transducer activity items (Fig. 2a, Fig. S2). The top 20 items for DE-lncRNA demonstrated that most biological processes involved metabolic processes (17/20, 85%) (Fig. 2b). The highest molecular function is binding including RNA, DNA, and protein binding (Fig. 2c). These results imply that DE-lncRNAs identified from the DMC patients are involved in transcription regulation.

We structured co-expression networks to determine if lncRNAs are associated with lncRNAs or mRNAs. There were 509 genes involved in our co-expression networks consisting of 117 lncRNAs and 392 mRNAs (Fig. S3), with 7 lncRNAs and 18 mRNAs harboring more than 10 related genes in the CNC network. The top 10 lncRNAs and mRNAs with the number of genes to which they are related are shown in Table 3. In this CNC network, lncRNAs ENSG00000279463, MSTRG.39819, and ENSG00000228063 were the top 3 lncRNAs with the greatest number of related genes (35, 20, and 18) (Fig. 2d-f). The related genes such as TOMM5, and MYLK have been reported to have implications in diabetes [19]; PDLIM1 and CAMK2G were found to be related to atherosclerosis [20]. We also analyzed the characteristics of 9,059 transcripts of novel lncRNAs identified in DMC patients. Most novel lncRNA transcripts harbored 2 exons (7060, 77.93%) (Fig. S4a). The lengths of most of the novel lncRNAs (71.91%) were less than 2000 bp (Fig. S4b). The conservation analysis in humans indicated that more than half (61.3%) had conservation scores (CS) less than 0.1 and 6.77% less than 0.01 between humans and other species (Fig. S4c). The distribution of chromosomal transcripts demonstrated that novel lncRNAs were primarily distributed on chr1 to chr6; and that most lncRNAs with low CS in humans were from the same chromosome (Fig. S5). These results reflect that lncRNAs in leukocytes may have an important effect on DMC.

Validation Measurement of DE-lncRNAs in expanding groups

To confirm the DE-lncRNAs, we independently detected 16 lncRNAs in the validation group of DMC patients (n = 46) and healthy controls (n = 36) via real-time PCR. The expression levels of these lncRNAs in the validation cohort are shown in Fig. 3a. Of the 16 tested lncRNAs, 12 lncRNAs (75%)

displayed dramatically different expression levels between the DMC and the healthy control groups; 4 lncRNAs were downregulated, and the others were upregulated in DMC. Nine novel lncRNAs and 3 known lncRNAs were positively confirmed. In addition, 8 lncRNAs belonged to the lincRNA, whereas 4 lncRNAs belonged to the antisense group (Table 4). Comparing the validation results and sequencing data showed that most lncRNAs (75%, 12/16) displayed similar trends, with 10 lncRNAs exhibiting the same significantly positive results as observed in the sequencing data (Fig.S6). Among the 12 significantly different expression levels of lncRNAs, 8 lncRNAs harbor predicted target genes (Table S5).

We sought to identify potential orthologs of our selected lncRNAs by comparing their sequences with previously identified murine lncRNAs. Of the 12 validation-positive lncRNA, 8 (66.67%) exhibited orthologous sequences in the mouse genome (Table 5). Thus, these validated lncRNAs exhibited comparable conservation in humans and mice, which could lead to the use of a mouse model to investigate lncRNA biomarkers directly.

DE-lncRNAs response to HG and IS conditions in EC

The pathological changes of DMC are primarily due to higher serum glucose levels inducing abnormal metabolism, consequently influencing the functions and homeostasis of endothelial cells [21-23]. A better understanding of lncRNAs response in EC under HG and IS is essential. Therefore, we tested the expression of 10 lncRNAs which are positively validated and exhibited the same significant trend as observed in the sequencing data within the HUVEC cell line. As shown in Fig. 3b and Fig.S7a, 3 lncRNAs exhibited increased levels after a 12 h and 24 h treatment with 25 nM, and 30 nM of glucose, respectively. lncRNA ENSG00000228063 was significantly downregulated after 24 h treated with HG in ECs (Fig. 3b, Fig.S7a).

Based on the “metabolic memory,” which is defined as a phenomenon that the vasculature can remember transient hyperglycemia for quite an extended period even after the reestablishment of normoglycemia [24], we tested if there was a metabolic memory of these 4 lncRNAs in ECs. The results illustrated that the lncRNA MSTRG.122492 and MSTRG.3528 were significantly upregulated, and ENSG00000228063 was dramatically downregulated (Fig. 3c). We also performed HO and IS treatment on ECs to mimic the environment of the pathological changes in DMC. The results showed that the expression of lncRNA MSTRG.3528 was significantly higher after a 12h HO stimulus, the others exhibited no change (Fig.S7b). The expression of lncRNA MSTRG.74858 and ENSG00000228063 markedly decreased after a 6h TNF- α treatment. MSTRG.3528 was downregulated after a 12h treatment with TNF- α . MSTRG.122492, ENSG00000269902, and MSTRG.159327 expression was downregulated after 6h and then reached normal levels after 12h (Fig. 4d). All the above results imply that ENSG00000228063 is the most sensitive in response to HG, HO, and IS.

lncRNA LYPLAL1-DT alleviates the influence of HG and IS on the proliferation, migration, autophagy, apoptosis, and inflammatory response of ECs

Based on the results of HG/IS test, and lncRNA ENSG00000228063 exhibited metabolic memory, we chose this transcript for further investigation. ENSG00000228063 is located on 1q41 LLP1 and is within 1 kb of LYPLAL1. Therefore, it was named LYPLAL1-DT (Ensembl ID: ENSG00000228063) (Fig. 4a). The major variant is 2,577 nt in length and encoded by five exons. LYPLAL1-DT is highly conserved in humans, with homologous sequences found only in chimpanzees, and other nonhuman primate animals (Fig.S7c), having a very low homology in mice. LYPLAL1-DT is expressed across diverse human tissues, including vascular tissue and ECs. As the position of the lncRNA is closely associated with its function, we first explored the location of LYPLAL1-DT in HUVEC cells. As shown in Fig. 4b, LYPLAL1-DT is expressed in both the nucleus and the cytoplasm, primarily in the cytoplasm, indicating that LYPLAL1-DT could exert cis-regulation or play a role as a competitive endogenous RNA (ceRNA).

To investigate the function of LYPLAL1-DT in ECs, we successfully constructed overexpressing HUVECs (OE) and their corresponding control cell line (OC) (Fig. 4C). The CCK8 staining results showed that LYPLAL1-DT significantly promotes the vitality of EC, especially under HG conditions (Fig. 4d). LYPLAL1-DT increases the proliferation of EC under an IS mimic via treatment with TNF- α (Fig. 4e). LYPLAL1-DT overexpression in EC alleviates migration under both HG and TNF- α treatment (Fig. 4f). These results reflect that LYPLAL1-DT overexpression effectively protects ECs under HG and IS by enhancing proliferation and migration.

Dysfunctional HUVECs promotes vascular inflammation by expressing inflammation cytokines and surface adhesion molecules involved in DMC development. We collected the media of EC-OE under HG and analyzed the concentrations of the secreted cytokines. The results illustrate that the inflammatory molecule IL-1 β (Fig. 4g) and the adhesion molecule ICAM-1 decreased (Fig. 4h); conversely, anti-inflammation molecules IL-10 and IL-13 were increased (Fig. 4i) in the EC-OE group and under HG conditions. Consistent with the high level of ICAM-1 in the cell culture media, the overexpression of LYPLAL1-DT significantly suppressed the adhesion of monocytic THP-1 cells onto the HG-treated HUVEC monolayers (Fig. 4j). These results suggest that LYPLAL1-DT plays a significant role in ameliorating the inflammatory conditions in ECs treated with HG.

LYPLAL1-DT affected SIRT1 expression by acting as a ceRNA sponging miR-204-5p

To explore the molecular mechanism of LYPLAL1-DT in the protection of EC, we were able to predict the miRNAs and the corresponding target genes using the bioinformatics tools TargetScan and miRcode. Upon analysis 13 miRNAs with a higher score were detected and found that 3 were upregulated and 2 were downregulated miRNAs in EC-OE compared to the control (Fig. S8). We chose miR-204-5p, the most significantly downregulated miRNA (Fig. 5a) in EC-OE of LYPLAL1-DT to investigate its function. The miR-204-5p mimic remarkably decreases the proliferation and migration of EC-OE (Fig. 5b, 5c). Subsequently, we constructed luciferase reporters with both a wild and mutant LYPLAL1-DT site used for miR-204-5p binding (Fig. 5d). The results demonstrated that the mimic miR-204-5p significantly suppresses the luciferase activity of wild type LYPLAL1-DT, compared to either the mutant or empty vector controls (Fig. 5e). We detected 9 target genes of miR-204-5p that were predicted using STARBASE

via real-time PCR (Fig. S9), then chose SIRT1 (Fig. 5f) and examined its expression level in EC-OE with and without the presence of the miR-204-5p mimic by real-time PCR and Western blot. SIRT1s expression was increased in EC-OE and conversely was decreased by the miR-204-5p mimic (Fig. 5g, 5h). Using a dual luciferase reporter test, it was demonstrated that SIRT1 is the binding targeted gene of miR-204-5p (Fig. 5i). Further results of the RIP assay showed that LYPLAL1-DT, miR-204-5p, and SIRT1 bind to Ago2 (Fig. 5j), confirming that LYPLAL1-DT functions as a competing endogenous RNA (ceRNA) regulating the expression and function of SIRT1 via inhibition with miR-204-5p. SIRT1 is an important gene related to autophagy and apoptosis, we detected the LC3B and apoptotic body under HG conditions. The data demonstrated that LYPLAL1-DT increases LC3 β / α levels, thus decreasing the number of TUNEL positive cells under HG conditions (Fig. 5k, 5l). Therefore, our results indicated that lncRNA LYPLAL1-DT inhibits miR-204-5p, consequently upregulating the expression of SIRT1. Furthermore, promoting autophagy, attenuating apoptosis levels in order to alleviate HG injury, and exerting protective effects on ECs.

LYPLAL1-DT from leukocytes may exert protective effects on HUVEC via exosome transportation

Exosomes regulate the biological functions of recipient cells via RNA transfer. Since LYPLAL1-DT was discovered in leukocytes, we hypothesized that LYPLAL1-DT was transmitted to HUVECs via exosomes. The serum exosomes of DMC patients and healthy control were extracted and RNA-sequencing was performed to confirm this hypothesis. The purified exosomes were detected by transmission electron microscopy (TEM) (Fig. 6a), NTA (Fig. 6b), and Western blot (Fig. 6c) using the positive exosome protein markers TSG101 and Alix. The RNA sequencing data showed that DE-lncRNAs (Fig. 6d, 6e) and DE-mRNAs (Fig. S10 a and b) were found between exosomes derived from DMC and control serum, with LYPLAL1-DT having the most significantly different expression levels of the lncRNAs, harboring trends similar to the results found in leukocytes (Fig. 6f). We also detected that the leukocyte marker CD11b+ is positively expressed in exosomes (Fig. 6c), proving that exosomes may originate from leukocytes. Thus, we treated HUVEC by exosomes from DMC patients' serum and healthy control and detected miR-204-5p level, cell viability and migration ability. As expected, miR-204-5p level was significantly lower in control than that in DMC group (Fig. 6g), while viability (Fig. 6h) and migration ability (Fig. 6i) of HUVEC were abbreviated in DMC group. This data confirmed that expression of LYPLAL1-DT in exosomes derived from the leukocytes could affected the ECs, protecting them from dysfunction.

Discussion

In the present study, we constructed the transcription profiles of circulating leukocyte lncRNAs and mRNAs in DMC patients and further selected and investigated the molecular mechanisms of a novel lncRNA LYPLAL1-DT having protective effects under pathological conditions. We identified 477 lncRNAs harboring significant differences between patients with diabetic macrovascular complications and healthy controls. This data is more than a microarray analysis of differentially expressed genes screened from the transcriptome of endothelial cells induced by high glucose [25]. Functional analysis and lncRNA-mRNA network analysis showed that it contained many genes that have been reported to be related to

diabetes and vascular diseases, such as TOMM5, MYLK, PDLIM1, and CAMK2G [26]. Next, we validated 12 lncRNAs in an expanding cohort. Pathological conditions that were adversely simulated showed that 4 lncRNAs were correlated with endothelial cell injury. Finally, lncRNA LYPLAL1-DT was selected and showed protective effects on endothelial cells by sponging to miR-204-5p as a ceRNA, thereby regulating the expression of SIRT1, enhance autophagy, and alleviating EC inflammation. These results strongly suggest that circulating lncRNAs in leukocytes can be an important target for further studies of diabetic vascular complications. Meanwhile, abnormal expression of lncRNA in leukocytes affects endothelial cells, including the adhesion of macrophages, similar to the reports that show lncRNA in the secreted bodies of endothelial cells can adjust the polarization effect of macrophages [27]. Upon further detection, it was also found that LYPLAL1-DT in exosomes from the serum of DMC patients are derived from leukocytes and is expressed at lower levels than in healthy controls and is similar in leukocytes. All results confirmed that lncRNAs in circulating leukocytes are crucial to the pathogenesis of DMC. Furthermore, LYPLAL1-DT is an important and potential new target for DMC research.

Endothelial cell stabilization is a pivotal event in the development of diabetes-associated vascular diseases [3]. Sustained high blood glucose in diabetes will continuously stimulate blood vessels, leading to endothelial injury, further stimulating mononuclear cells to accumulate within the blood vessel wall; thus, accompanied by inflammation, and eventually leading to the occurrence of cardiovascular disease [28]. lncRNAs have emerged as critical regulators in a variety of EC biological or pathological processes. Yan B et al. reported that lncRNA-MIAT is involved in angiogenesis and therapeutics against neovascular diseases [29]. lncRNA MALAT1 is enriched and regulates migration and vascular sprouting of ECs [12]. In this study, ECs were induced by hyperglycemia and inflammatory factors in order to simulate the development of diabetic macrovascular complications. From 12 positively validated lncRNAs in an expanded cohort, 4 lncRNAs were found to change during these treatments. Furthermore, we used a metabolic memory test and found a novel lncRNA LAPLAL1-DT that is sensitive during the stimulating condition as well as returning to normal blood sugar levels, known as hyperglycemic “metabolic memory” in diabetes-related complications [30]. It is reported that eNOS mediates metabolic memory leading to continuous aortic inflammation and endothelial dysfunction [31]; however, evidence of lncRNA involved in EC-metabolic memory is still limited. In this study, we confirmed that lncRNAs in endothelial cells harbor the phenomenon of hyperglycemic metabolic memory. Furthermore, we found that overexpression of LYPLAL1-DT could decrease the adhesion of monophages onto the surface of ECs, enhancing the secretion of anti-inflammatory cytokines IL-10 and IL-13, which is further evidence of the protective effects of EC [32]. These findings suggest that lncRNAs are involved regulating endothelial cells in the case of diabetes-induced vascular injury and are an important member in the regulation of diabetic macrovascular complications.

In recent years, accumulating evidence highlights a growing list of lncRNAs related to glucose homeostasis and diabetic complications. The molecular mechanisms of lncRNAs can be used as ceRNA in order to regulate target genes by acting as miRNA inhibitors, thus regulating the expression of protein-coding genes [33]. CeRNA plays a vital role in regulating the expression of hyperglycemic response genes. For example, lncRNA ca7-4 ceRNA of miR877-3p and miR5680 promotes autophagy and apoptosis of

vascular endothelial cells induced by high glucose [11]. In our study, novel lncRNA LYPLAL1-DT was found to regulate the target gene SIRT1 by acting as a ceRNA of miR-204-5p. SIRT1 is an anti-autophagy factor that can reduce cell death [34], as well as regulate the apoptosis of endothelial cells through the mTOR pathway [35]. It is one of the most important roles in vascular biology and atherogenesis [36]. For instance, SIRT1 can also protect endothelial cells from high glucose induced injury [34]. In the current study, we found SIRT1 is the target gene of LYPLAL1-DT, therefore elevates autophagy levels, thus decreasing apoptosis, inflammation, and monocyte adhesion of ECs. These results are similar to previous reports that SIRT1 inhibits monocyte adhesion to the vascular endothelium [37], and is involved in the inhibitory effects of endothelial cell apoptosis [38]. More importantly, we confirmed the most recent report that miR-204-5p/SIRT1 mediated inflammation and apoptosis in endothelial cell dysfunction under cyanidin-3-O-glucoside treatment[39]. Combined with the report that SIRT1/FoxO1 pathway enhances autophagy flux in order to prevent atherosclerosis and arterial thrombosis [40], we believe SIRT1 is the critical role in DMC. Hence, we present evidence that LYPLAL1-DT effectively protects against vascular endothelial injury in diabetes associated complications via the LYPLAL1-DT -miR-204-5p/SIRT1 pathway.

There are still some limitations in our study. First, the number of expanded samples for verification is not very large, primarily due to our relatively strict enrollment conditions. We only accepted patients who were initially diagnosed with having diabetic macrovascular complications. Second, how lncRNAs from leukocytes regulate endothelial cell function is still largely unknown, although we found that lncRNA LYPLAL1-DT is also present in serum exosomes, with a similar downregulation trend as in leukocytes. These exosomes positively expressed leukocytic markers, partly demonstrates that leukocytes are the main source of serum exosomes. Moreover, we primarily confirmed that exosome with lower LYPLAL1-DT could abbreviate viability and migration of EC. Finally, this study lacks further research on the function of LYPLAL1-DT using an animal model due to the very low conservation of LYPLAL1-DT between humans and mice, as shown in Table 5 and Fig. S7. The species-specific effect of LYPLAL1-DT in DMC remains to be further elucidated. Nevertheless, our observations underscore the importance of LYPLAL1-DT in DMC using human cells.

Conclusion

In general, We identified 12 DE-lncRNAs related to DMC, among which lncRNA LYPLAL1-DT was identified to be transmitted from leukocytes to EC via exosomes and have protective effects on EC as ceRNA mediated through the miR-204-5p/SIRT1 pathway. Therefore, it inhibits the autophagy of EC as well as modulating systemic inflammation. This approach could be regarded as a new potential therapeutic target in DMC.

Abbreviations

lncRNA: long non-coding RNA

DMC: type 2 diabetes with macrovascular complication

EC: endothelial cell

HG: high glucose

NG: normal glucose

IS: inflammatory conditions

DE: differentially expressed

HO: hypoxia

ceRNA: competing endogenous RNA

LYPLAL1-DT: LYPLAL1 divergent transcript

SIRT1: sirtuin 1

IL-6: Interleukin 6

TNF- α : tumour necrosis factor alpha-like

WT: wild type

MUT: mutant type

OC: corresponding control

OE: over-expression

RIP: RNA Immunoprecipitation Assay

Declarations

Ethics approval and consent to participate

This study was conducted on the basis of the Declaration of Helsinki, after obtaining the written informed consent of the participating patients and the approval of the Ethics Committee of Chinese PLA General Hospital (Permitted No. S2016-147-03).

Consent for publication

Once the manuscript is accepted, we approve it to be published in Cardiovascular Diabetology.

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request

(The sequencing data of DMC is in the process of uploading to GEO).

Competing interests

The authors declare that there is no conflict of interest associated with this manuscript.

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Authors' Contribution

All authors were responsible for drafting the article and revising it for important intellectual content. They all approved the version to be published.

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Figures

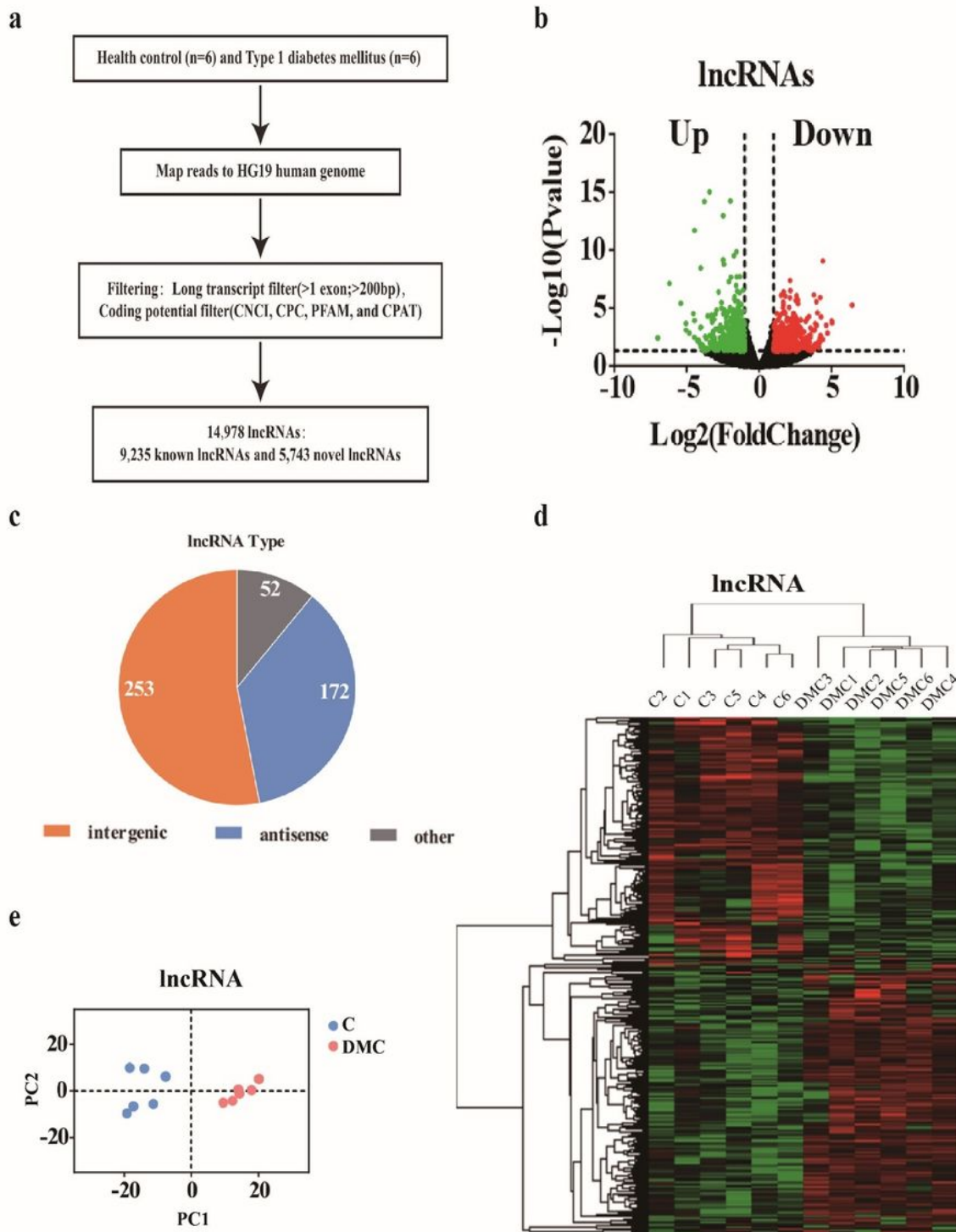


Figure 1

Characteristics of lncRNAs transcription in type 2 diabetes macrovascular complication. (a) A schematic illustration of the procedure to identify and define lncRNAs in the leukocytes of DMC patients. (b) Differentially expressed lncRNAs were identified from a Volcano plot showing data from DMC diabetes patient relative to healthy controls. The vertical black lines correspond to 2-fold up and downregulations, respectively; and the horizontal black line represents a p-value of 0.05. The red and green points in the

plots represent the differentially expressed genes with statistical significance for upregulation and downregulation of lncRNA, respectively. (c) Pie chart representations show the proportion of DMC lncRNAs that are transcribed as antisense (blue), intergenic (orange) or other types (grey). (d) Differential lncRNA expression profiles were hierarchical cluster analyzed and shown as a heatmap. (e) Principal component analysis shows similar results also presented as a heatmap.

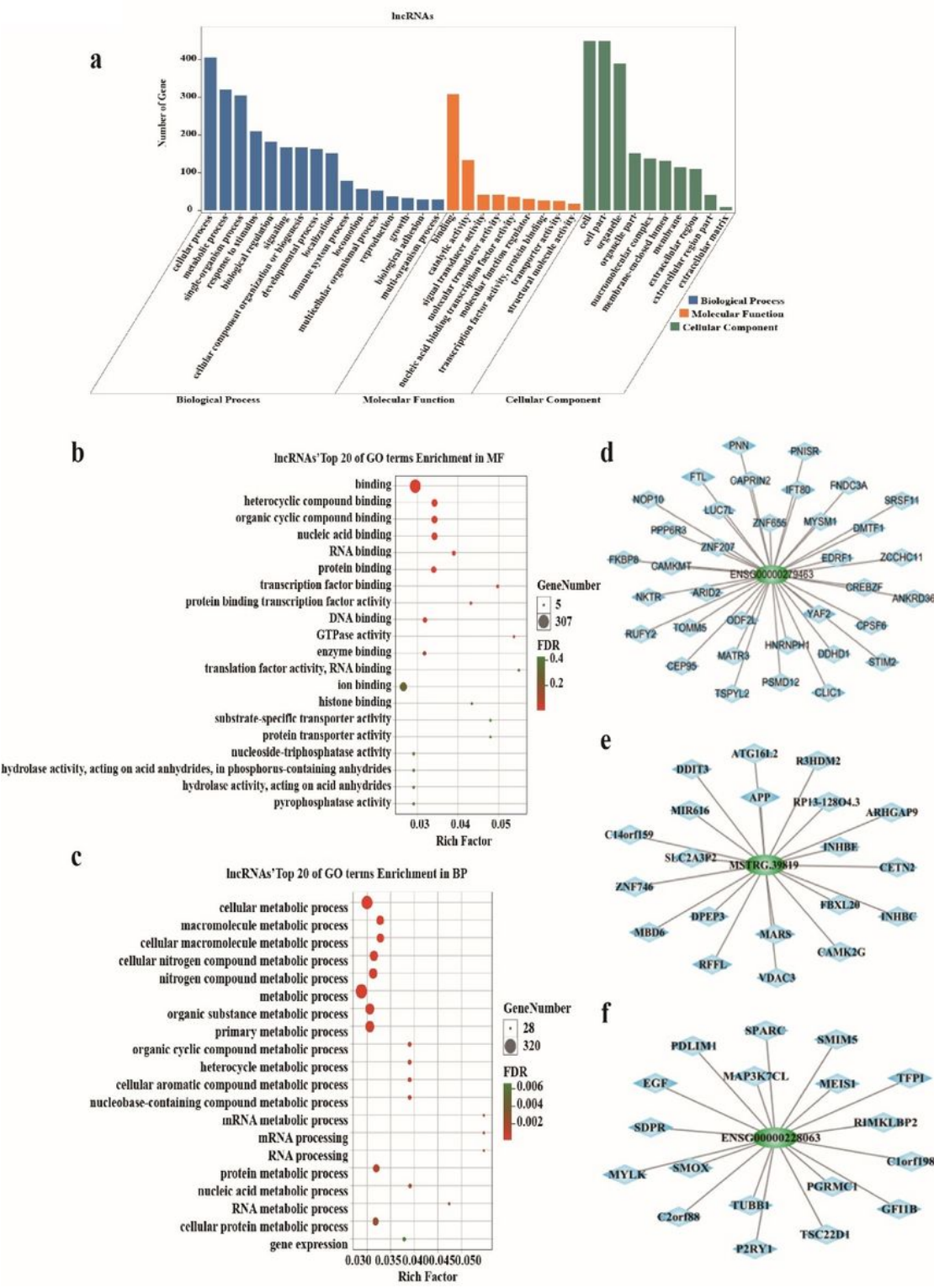


Figure 2

Bioinformation analysis by enrichment analysis of GO terms for DMC-lncRNAs and co-expression networks for DMC-lncRNAs and mRNAs. (a) GO analysis of DMC-lncRNAs in biological processes, molecular functions, and cellular components. (b) The top 20 GO terms of lncRNAs enriched in biological processes. (c) The top 20 GO terms of lncRNAs enriched in molecular function. (d, e, f) The co-expression network of 3 lncRNAs including ENSG00000279463, MSTRG.39819 and ENSG00000228063.

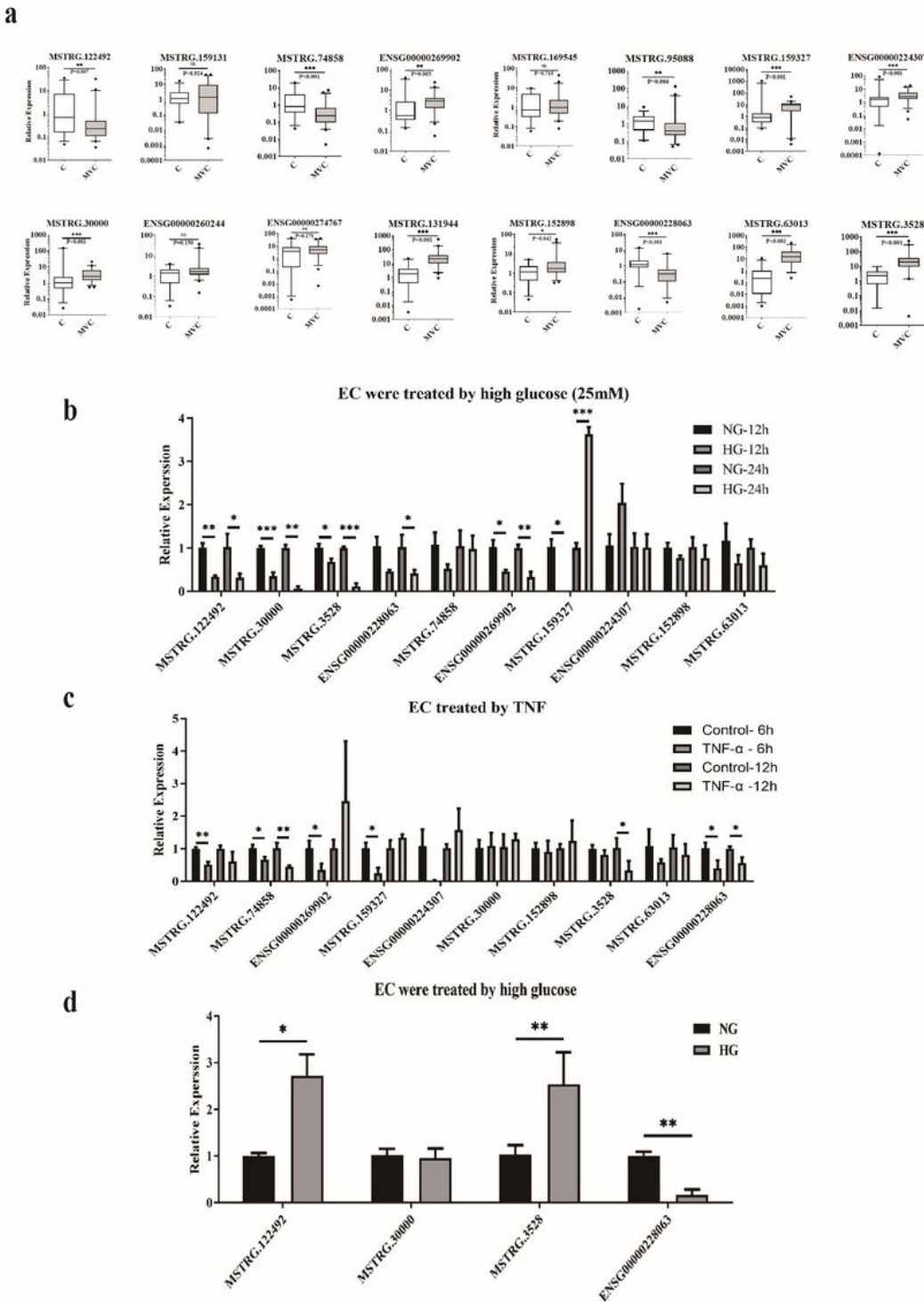


Figure 3

Validation of lncRNAs in DMC patients and lncRNAs expression levels induced by high glucose and TNF- α in HUVEC. (a) There are 12 of 16 lncRNAs showed significant difference in expression levels between DMC patients (n = 46) and healthy controls (n = 36) by real-time PCR. (b) The lncRNAs expression in HUVEC after treated with 25mM glucose for 12h and 24h. (c) The lncRNAs expression in HUVEC after treated with 25mM glucose and then resuming normal culture for 72h. (d) The lncRNAs expression in HUVEC after induced by TNF- α for 12h and 24h. "NS": no significant difference; "*" indicated significant difference with p<0.05, "**" indicated p<0.01 and "***" indicated p<0.001.

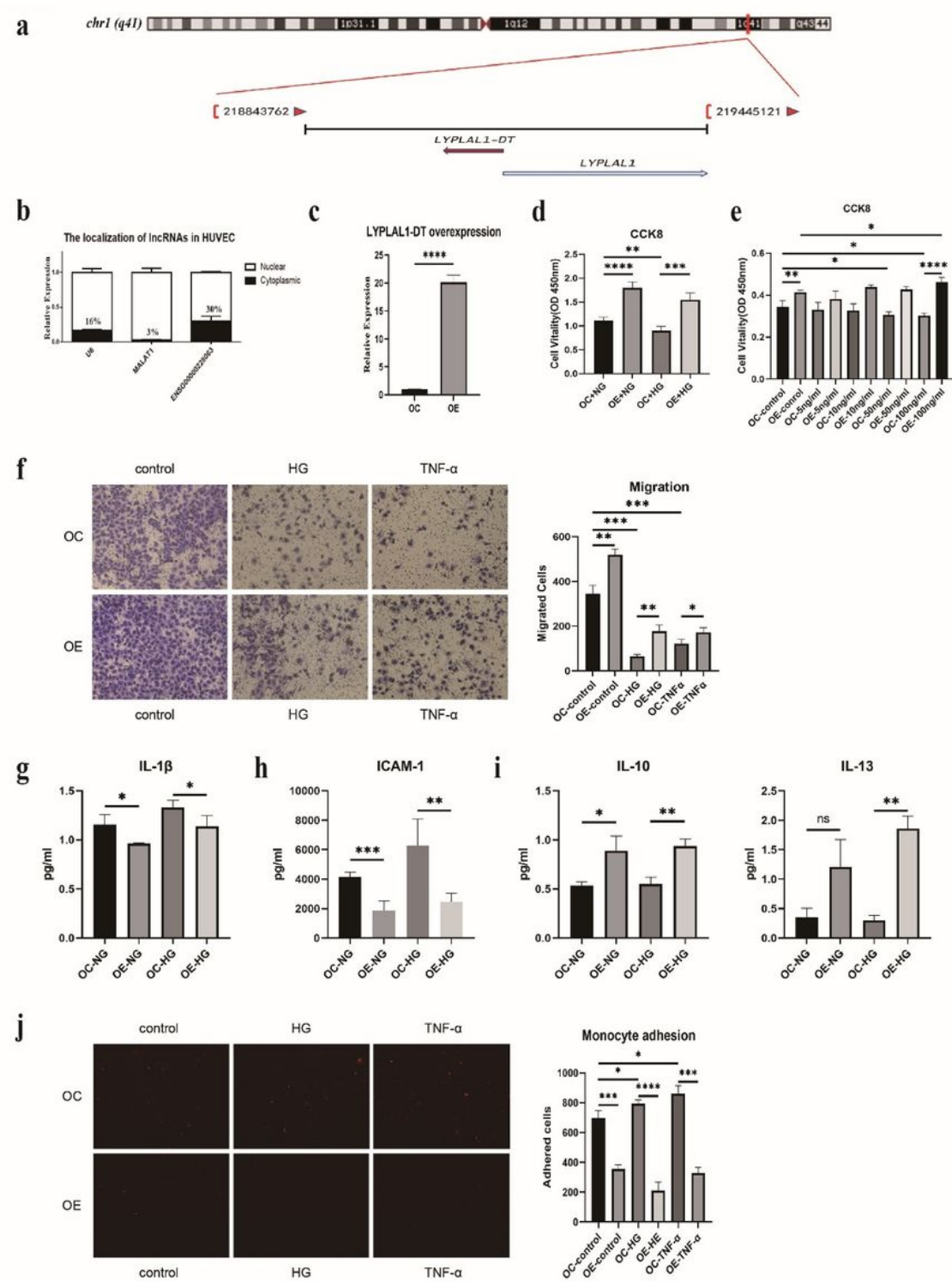


Figure 4

The location of LYPLAL1-DT and the biological function of LYPLAL1-DT in HUVEC. (a) The location of LYPLAL1-DT on the chromosome. (b) The Subcellular localization of LYPLAL1-DT in HUVEC. (c) Establishment of LYPLAL1-DT-overexpression cell line with a pLenti-GIII-CMV-CBH-GFP-2A-Puro vector being transfected into HUVEC was identified by real-time PCR. (d, e) CCK-8 assays were performed to determine the viability of LYPLAL1-DT-overexpression cells treated with HG (25mM) or induced by TNF- α for 12h. (f) Transwell assays were used to investigate the migration of LYPLAL1-DT-overexpression cells treated with HG (25mM) or induced by TNF- α (100ng/ml) for 12h. (g, h, i) The HG-treated and TNF- α -induced cell culture medium were collected to detect the expression levels of cytokines, including IL-1 β , ICAM-1, IL-10, IL-13, using ELISA Kits. (j) LYPLAL1-DT-overexpression HUVECs were pretreated with HG or TNF- α as described above, then adhered Dil-stained THP-1 cells were visualized by fluorescence microscopy. “*” indicated significant difference with $p < 0.05$, “**” indicated $p < 0.01$ and “***” indicated $p < 0.001$.

Fig. 5

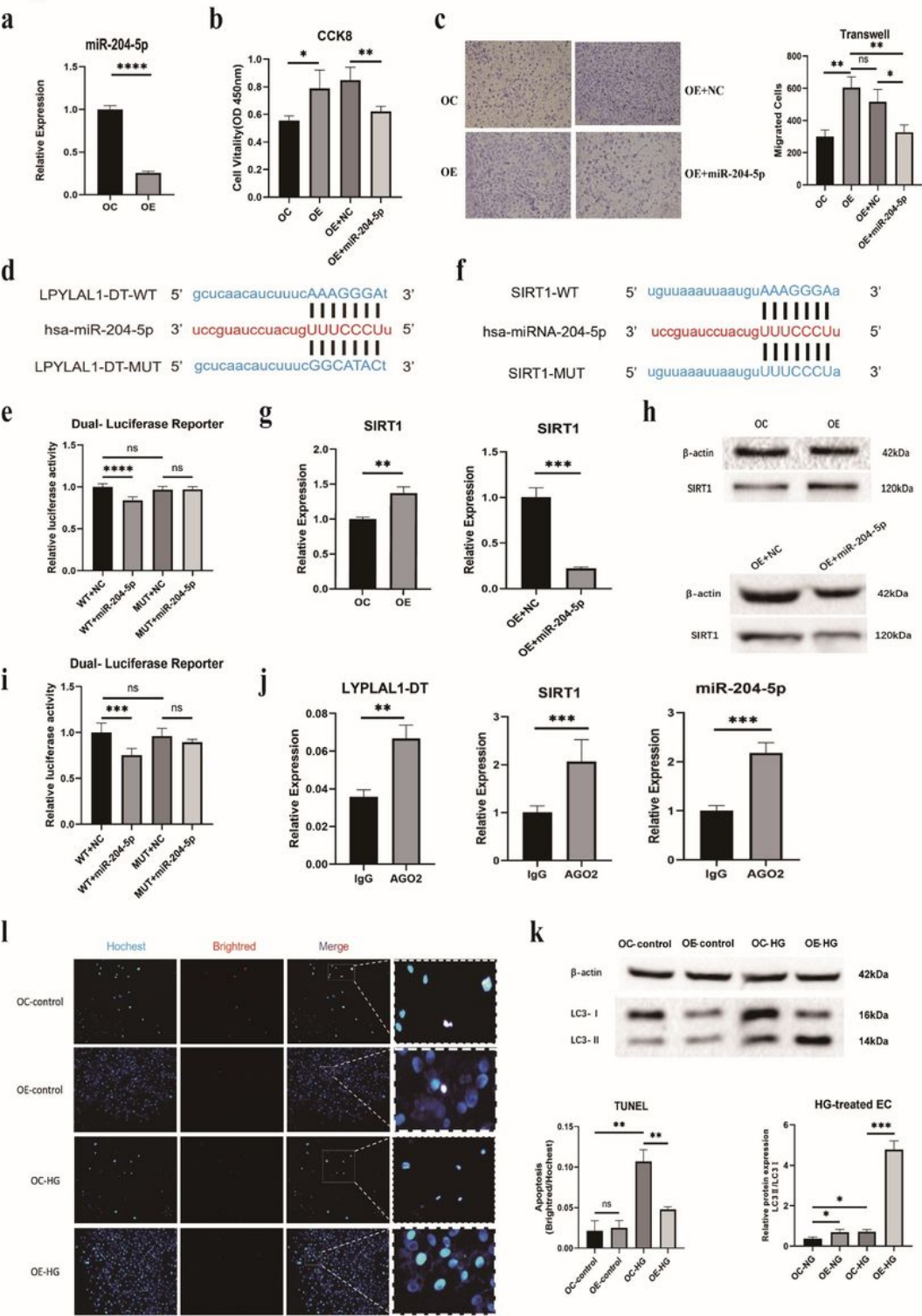


Figure 5

LYPLAL1-DT functions as a sponge and competes with SIRT1 to bind miR-204-5p. (a) Real-time PCR was used to identify the expression level of miR-204-5p in LYPLAL1-DT-overexpression cells. (b, c) CCK-8 and transwell assays were performed to evaluate the proliferation and migration of EC-OE transfected with miR-204-5p mimic. (d, f) The binding sites of miR-204-5p in LYPLAL1-DT and SIRT1 3'UTR were predicted by TargetScan. (e) Luciferase reporter plasmid containing wildtype (WT) or mutant (MUT) LYPLAL1-DT

was co-transfected with miR-204-5p mimic into HUVEC. (g, h) The SIRT1 expression level was identified by real-time PCR and Western Blot in LYPLAL1-DT-OC/OE and LYPLAL1-DT-OE transfected with miR-204-5p mimic. (i) Luciferase reporter plasmid containing wildtype (WT) or mutant (MUT) SIRT1-3'UTR was co-transfected with miR-204-5p mimic into HUVEC. (j) RIP assays were performed in HUVEC and the RNA co-precipitated with Ago2 was used to quantify the LYPLAL1-DT, miR-204-5p and SIRT2 expression levels by real-time PCR. (k) The LYPLAL1-DT-OC/OE treated with HG (25mM) for 12h were collected to detect the autophagy-associated protein LC3 $\bar{\bar{}}$ and $\bar{\bar{}}$. (l) LYPLAL1-DT-OC/OE were treated with HG (25mM) for 12h and then were used to inspect apoptosis via TUNEL.

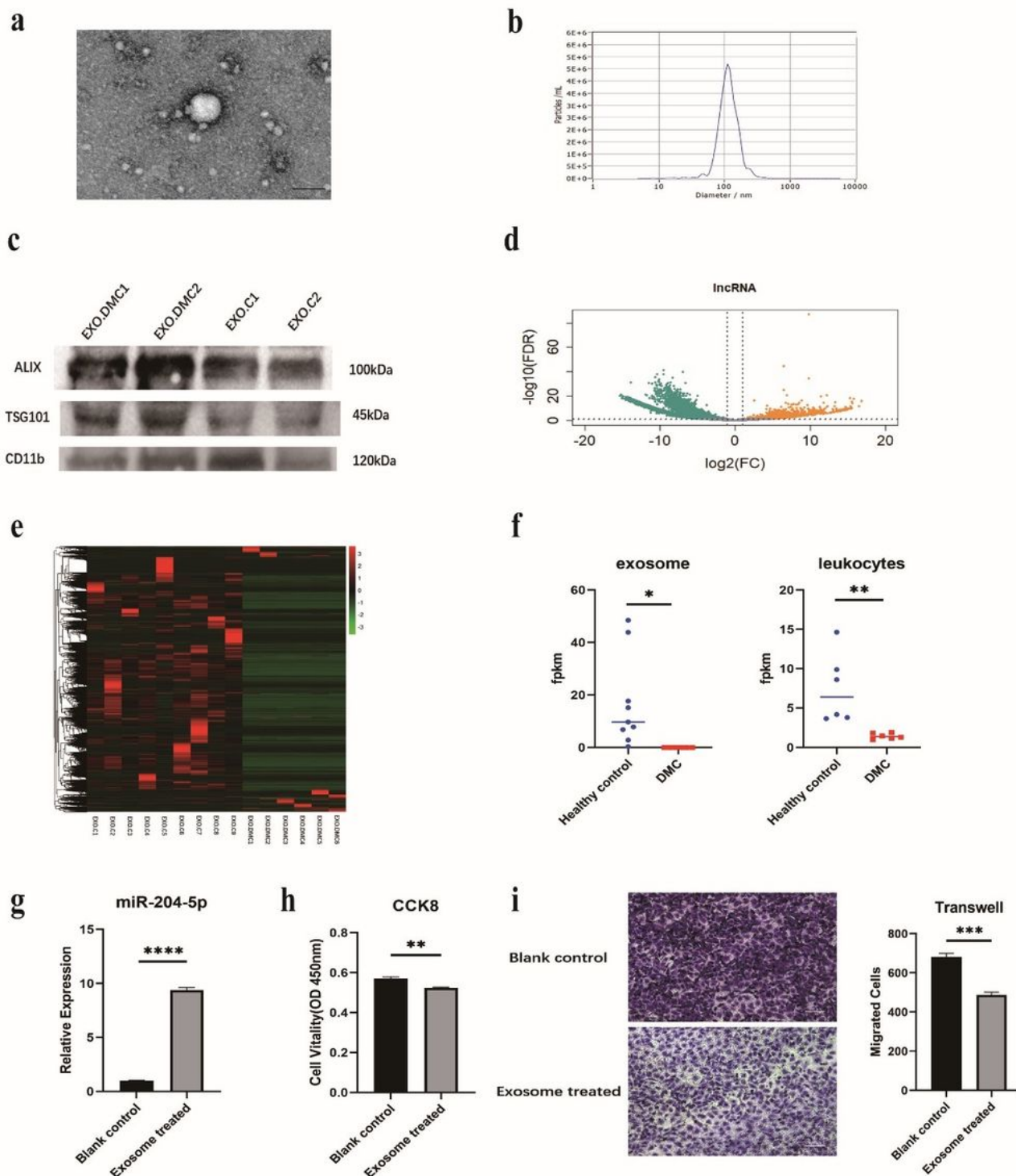


Figure 6

LYPLAL1-DT was probably transferred from Leukocytes to HUVEC via exosomes in DMC patients. (a) Morphology of leukocytes-derived exosomes was shown by TEM. (b) Leukocytes-derived exosomes were observed to be between 30-120 nm in size by NTA. (c) Western Blot was used to examine exosome marker ALIX and TSG101, and leukocyte marker CD11b. (d, e) Differentially expressed lncRNAs were identified from a Volcano plot and hierarchical cluster analyzed, shown as a heatmap. (f) RNA-

sequencing of DMC patients-derived exosomes and DMC patients' leukocytes shows a similar trend of LYPLAL1-DT expression level. (g) The expression of miR-204-5p in HUVECs increased when treated with exosomes derived from DMC patients' serum compared with control. Exosomes derived from DMC patients' serum significantly abbreviate viability and migration of HUVEC compared with control detected by (h) CCK8 and (i) Transwell assays.

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