Downregulation of Exosomal miR-26a-5p Promotes Lymphangiogenesis and Lymphatic Metastasis in Endometrial Cancer

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Research

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Abstract

Background: Endometrial cancer (EC) patients with lymph node (LN) metastasis have poor prognosis. However, the potential biomarkers that predict LN metastasis and the molecular mechanism of tumor-induced peritumoral lymphangiogenesis have not been well explored. Cancer-secreted exosomal miRNAs are emerging mediators of cell-cell communication in the tumor environment.

Methods: Exosomes were isolated with a differential centrifugation method and confirmed by Transmission electron microscopy, NanoSight analysis, and Western blot. MicroRNA (miRNA) sequencing of exosomes derived from EC patients and healthy donors were performed. FISH and qRT-PCR were used to detect the indicated miRNA expression. Exosomal miRNA transferred to cells were confirmed by immunofluorescence and confocal microscope. siRNA and plasmid transfections as well as viral infection were performed to manipulate gene expression. A series of in vitro and in vivo phenotype experiments (tube formation, migration, and popliteal LN metastasis model) were performed to investigate the role of indicated miRNA in EC. RNA sequencing was used to select the underlying transcription factor, and luciferase activity assay and chromatin immunoprecipitation were performed to elucidate the molecular mechanisms.

Results: Our data showed that serum exosomal miR-26a-5p was significantly reduced in EC patients, and its level was positively associated with LN metastasis. Loss of miR-26a-5p promoted the migratory and invasive abilities of EC cells, and miR-26a-5p could be transferred from EC cells-secreted exosomes into human lymphatic endothelial cell (HLEC). Mechanistically, miR-26a-5p could regulate LEF1/c-myc/VEGFA axis via binding to its direct downstream target lymphoid enhancer binding factor 1 (LEF1), consequently promoting HLEC tube formation and migration in vitro, facilitating lymphangiogenesis and LN metastasis in vivo. Re-expression and knockdown of LEF1 could respectively promote and rescue the effects induced by exosomal miR-26a-5p. Moreover, we demonstrated that transcriptional factor EB (TFEB) directly induced miR-26a-5p expression.

Conclusions: Our results show that exosomal miR-26a-5p/LEF1/c-myc/VEGFA axis is dysregulated and plays a critical role in LN metastasis and exosomal miR-26a-5p may be used as a blood-based biomarker for EC patients with LN metastasis.

Background

Endometrial cancer (EC) is one of the most common gynecologic cancer worldwide, with an estimated 66,570 new cases and 12,940 deaths in 2021 [1]. Lymph node status is one of the most important prognostic factors. Patients with early EC, whether lymphadenectomy must to be performed and its extent are still controversial. In addition, EC patients with lymph node (LN) metastasis have extremely poor prognosis, which decreases the 5-year survival rate from 95% to 69% [1]. Approximately 20% of EC patients are diagnosed with regional metastasis. Despite the overwhelming evidence for the role of LN metastasis in cancer, the specific biomarker and the definite molecular mechanism that drives it in EC
remain unclear. Thus, it is of great value to elucidate the mechanism of LN metastasis and identify biomarkers that could be used for diagnosis, prognosis, and intervention of cancer metastasis.

Lymphangiogenesis is the process of growing new lymphatic vessels, which involves proliferation, sprouting, and migration of lymphatic endothelial cells [2]. Increased density of tumor-related lymphatic vessels is closely associated with LN metastasis and poor prognosis [3]. Multiple lines of evidence indicated that peritumoral lymphatic vessels facilitated the spread of tumor cells from their primary site to regional LNs [2, 4]. In addition, the inhibition of cancer-induced lymphangiogenesis has been proposed as a means of blocking the metastatic spread of cancer [5]. However, the potential biomarkers that predict LN metastasis and the molecular mechanism of tumor-induced peritumoral lymphangiogenesis have not been well explored.

Exosomes are small membrane vesicles ranging 30-150 nm in size that are secreted into the extracellular environment by multiple types of cells, including cancer cells, [6]. Due to its stability in body fluids, exosomes could be used in a variety of cancer diagnosis and clinical application. Expression patterns of exosomal miRNAs reflect the dysregulated miRNAs in cancer cells to a certain extent [7]. Moreover, cancer cell-derived exosomal miRNAs can be transferred into recipient normal host cells, which were identified as pivot messengers in cell-cell communication associated with the regulation of target genes [8, 9]. Therefore, cancer-derived exosomal miRNAs were crucial molecules in regulating the biological functions of recipient cells.

In this study, we identify that miR-26a-5p was downexpressed in EC tissues and lowly enriched in EC-derived exosomes from patients with EC, and associated positively with LN metastasis. Transcription factor EB (TFEB) could induce miR-26a-5p expression by interacting with the miR-26a-5p promoter region. In addition, we demonstrate that EC cell-derived exosomal miR-26a-5p can be transferred to human lymphatic endothelial cell (HLEC) and thereby promotes lymphangiogenesis by targeting lymphoid enhancer binding factor 1 (LEF1). Finally, our findings highlight a novel molecular mechanism of exosomal miR-26a-5p-mediated LN metastasis and identify miR-26a-5p from circulating exosomes of EC patients as a blood-based biomarker for prediction of LN metastasis.

Methods

Exosomes isolation and identification

Exosomes were isolated from EC-derived conditioned media or serum of EC patients according to the protocol of exoRNeasy serum kit (Qiagen, Hilden, Germany) with a differential centrifugation method. EC cells were cultured in DMEM/F12 medium supplemented with 10% exosome-depleted fetal bovine serum. The pelleted exosomes were subjected to transmission electron microscopy, nanoparticle tracking analysis, RNA extraction, protein assay, in vitro cell treatment, or in vivo administration. The amount of exosomes was measured by the BCA Protein Assay kit (Qiagen). For RNA extraction from exosomes, we used miRNeasy Mini Kit (Qiagen). For exosomes labeling, exosomes were fluorescently labeled with PKH67 membrane dye (Sigma). Labeled exosomes were resuspended in 10 ml phosphate-buffered saline.
(PBS), collected by ultracentrifugation. For in vitro cell treatment, 2 μg of exosomes were added to 2 × 105 recipient cells for 48 h. For in vivo administration, 10 μg of exosomes resuspended in 20 μl PBS were injected into the center of the xenograft tumors once every three days.

**Next-generation sequencing exosomal miRNA analysis**

We collected 8 serum samples from 4 cases of EC patients and 4 cases of healthy donors, and then exosomes were extracted from serum. Then, total RNA, containing miRNA, was extracted from serum exosomes using the miRNeasy Mini kit (Qiagen, Hilden, Germany). RNA samples were sent to Guangzhou Gminix, Biotechnology Co, Ltd. (Shanghai, China) for miRNA next-generation sequencing on an Illumina HiSeq 2500 sequencing platform with 10 M reads (Illumina, San Diego, CA).

**Cell lines and human tissue specimens**

Human lymphatic endothelial cell (HLEC), normal endometrial epithelial cell (EEC), human endometrial cancer cell lines Ishikawa (ISK), HEC-1B, HEC-1A, KLE, AN3CA were all purchased from the American Type Culture Collection (ATCC). HLEC was cultured in ECM medium (ScienCell, CA, USA) supplemented with 5% fetal bovine serum (HyClone, Logan, USA). EEC, ISK, HEC-1B, HEC-1A, KLE, and AN3CA cell lines were cultured in DMEM/F12 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, USA). A total of 50 cases of EC tissue specimens, corresponding to 30 cases without LN metastasis and 20 cases with LN metastasis, were collected from EC patients who underwent radical surgery without prior radiotherapy and chemotherapy between 2018 and 2019 at the International Peace Maternity and Child Health Hospital. A total of 50 cases of serum samples were collected from EC patients and 50 cases of serum samples were collected from healthy donors. Informed consent was obtained from all patients, and the research was approved by the Ethics Committee of International Peace Maternity and Child Health Hospital.

**Stable transfection with lentiviral vector**

Lentivirus vectors expressing miR-26a-5p and repressing miR-26a-5p were constructed and generated by Genechem Inc. HEC-1B was transfected with LV-Fluc-miR-26a-5p-up and its negative control vector (LV-Fluc-miR-26a-5p-up-NC). ISK was transfected with LV-Fluc-miR-26a-5p-down and its negative control vector (LV-Fluc-miR-26a-5p-down-NC).

**RNA interference and plasmids**

Inhibitor and mimic of miR-26a-5p were purchased from RiboBio Inc. The LEF1-coding sequence (without 3’-UTR) was cloned into pCDNA3.1(+)-vector. The empty vector was used as a blank control. In the rescue experiments, cells that stably expressed miR-26a-5p or incubated with exosomal miR-26a-5p were transfected with the human LEF1 expressing plasmids (OBiO Technology, Shanghai). TFEB expressing plasmids and siTFEB were designed and synthesized by OBiO Technology. Lipofectamine 3000 Reagent (Invitrogen) was then used to transfect siTFEB, human TFEB expressing plasmids, and human LEF1 expressing plasmids according to the manufacturer's protocol.
Fluorescence in situ hybridization and Immunofluorescence

Formalin-fixed paraffin-embedded sections were cut into 4.0-μm sections of tumor specimens. FISH was performed in tumor sections using fluorescence in situ hybridization (FISH) kit (Bosterbio, USA) and the miR-26a-5p detection probe (Boster, Wuhan, Chian) by following the manufacturer's protocol. For immunofluorescence, serial 4.0-μm paraffin section from EC tissues were analyzed by immunofluorescence with the Opal 4-color kit (PerkinElmer) according to the manufacturer's protocol.

Bioinformatic miRNAs target prediction

Two online programs TargetScan and miRDB were used to predict potential target genes for miR-26a-5p. GeneCards website (http://www.genecards.org) was used to identify relative regulators of endometrial cancer and lymphatic metastasis.

Quantitative real-time-PCR

Total RNA was extracted from cell lines and human EC tissues using the TRIzol Reagent (Invitrogen, USA), and miRNA was extracted by the RNeasy/miRNeasy Mini kit (Qiagen). Reverse transcription was performed using PrimeScriptT reagent kit (Qiagen), and the ABI Prism 7000 Sequence Detection System (ABI 7000 SDS) was used for real-time PCR analysis. U6 was used as the endogenous control of miRNAs, and GAPDH gene was used as an internal control for other mRNAs. Each experiment was repeated three times.

Western blotting

Total protein from cells and exosomes were prepared in RIPA buffer with protease inhibitors and quantified using BCA assay kit (Thermo Fisher Scientific Inc., MA, USA). Subsequently, protein lysates were resolved by SDS-PAGE, transferred onto PVDF membranes (Millipore, Bedford, MA). The antibodies used in the experiments included anti-β-catenin monoclonal antibody (1:1000, Cell Signaling Technology), anti-LEF1 monoclonal antibody (1:1000, Cell Signaling Technology), anti-c-myc monoclonal antibody (1:1000, Cell Signaling Technology), anti-VEGFA monoclonal antibody (1:1000, Abcam), anti-TFEB monoclonal antibody (1:1000, Cell Signaling Technology), anti-PI3K monoclonal antibody (1:1000, Cell Signaling Technology), anti-p-PI3K monoclonal antibody (1:1000, Cell Signaling Technology), anti-AKT monoclonal antibody (1:1000, Cell Signaling Technology), anti-p-AKT monoclonal antibody (1:1000, Cell Signaling Technology), and anti-GAPDH (1:5000, Santa Cruz). Each experiment was repeated three times.

HLEC tube formation assay and transwell migration assay

For tube formation assay, matrigel matrix (Corning) was plated in 48-well plate and incubated at 37 °C for 30 min to allow the matrigel to form a solid structure in the bottom. The treated HLECs were seeded onto the matrigel-coated well. After 12 h incubation, the plate was observed for the tubular structure with microscope. The tube length was quantified by ImageJ software (National Institutes of Health, Bethesda, USA). Each experiment was repeated three times. For transwell migration assay, 1 × 105 cells in 200 μl
ECM medium without FBS were seeded on a fibronectin-coated polycarbonate membrane insert in a Transwell apparatuses (Corning), and 600 μl medium with 10% FBS was added to the lower chamber. After 24 h of incubation, the cells invaded to the bottom of the inserted membrane were fixed with methanol for 15 min and then stained with 0.1% crystalline violet solution. We counted the cell numbers for analysis under a microscope in five random fields.

**Chromatin immunoprecipitation (ChIP)**

Cells were fixed with 1% formalehyde for 10 min at room temperature. Then, the cells were washed twice with PBS at 4 °C, collected and resuspended in lysis buffer and lysed on ice for 30 min. Cells were sonicated 5 times for 5 s to solubilize and shear cross-linked DNA. The chromatin (25μg) was immunoprecipitated for 12 h with 2 μg of anti-TFEB antibody or IgG. After incubation, protein G magnetic beads were then washed for 5 min with buffers. The immune complexes were eluted with elution buffer. After RNase A and proteinase K treatments and reversal of cross-linking, DNA was obtained by phenol and phenol/chloroform extractions. PCR amplifications of the precipitated DNA were carried out.

**Luciferase activity assay**

The putative miR-26a-5p complementary site in the 3′-UTR of LEF1 or its mutant sequence was cloned into the pMIR-REPORT Luciferase vector (OBiO Technology, Shanghai, China). 293T cells were co-transfected with the pMIR-REPORT-LEF1-3′UTR-WT or pMIR-REPORT-LEF1-3′UTR-MT vector and miR-26a-5p mimic/NC. And, cells were seeded into a 24-well plate and co-transfected with luciferase reporter constructs encoding the wild-type 3′-UTR region of miR-26a-5p or a mutated miR-26a-5p 3′-UTR region and TFEB plasmid using Lipofectamine 3000 (Invitrogen, California, USA). Luciferase activity was measured 48 h after transfection and analyzed by using a Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase signal was normalized to Renilla luciferase signal.

**Popliteal LN metastasis model**

NOD-SCID mice (5 weeks old) were purchased from the Experimental Animal Center, Shanghai Jiaotong University School of Medicine (Shanghai, PR China) and were used for the lymphatic metastasis model. The studies were approved by the Institutional Animal Research Ethics Committee of Shanghai Jiaotong University School of Medicine. Luciferase-labeled HEC-1B cells (1 × 107) were injected into the footpad of the mice. Then, the mice were randomly divided into three groups (n = 5 per group) and injected intratumorally with PBS, HEC-1B-exo\textsubscript{vector}, or HEC-1B-exo\textsubscript{miR}(20 μg per dose) every 3 days. Lymphatic metastasis was analyzed using a IVIS® Spectrum In vivo Imaging System (Xenogen Corporation). The footpad tumors and popliteal LN were excised when the tumors reached a comparable size 150 mm3 (volume = length × (width)2 / 2). FISH and IHC were used to analysis the sections of primary tumors and popliteal LN.

**Statistical analysis**
GraphPad Prism 6 software was used for statistical analysis. Quantitative values were presented as the mean ± SD. Differences among/between groups were analyzed by one-way ANOVA or Student’s t-test. The χ²-test was used for categorical variables. The degree of linear relationship between the expression levels of exosomal miR-26a-5p and miR-26a-5p in endometrial cancer lesion was analyzed by pearson's correlation coefficient. Significant difference was indicated by $P < 0.05$. Adobe Illustrator CC, Adobe Photoshop CC, and Image J software were used for figure presentation.

Results

Downregulation of exosomal miR-26a-5p positively correlated with LN metastasis in EC patients

To identify potential exosomal miRNAs in EC, next-generation sequencing (NGS) was performed using serum exosomes from four healthy donors and four patients with EC (Fig. 1A). Among the differently expressed miRNAs, miR-183-5p, miR-182-5p, miR-516b-5p, miR-411-3p, miR-873-5p, miR-15a-5p, miR-101-3p, miR-26a-5p, miR-21-5p, and miR-199a-3p were the top ten significantly dysregulated in EC serum exosomes compared with those from healthy donors (Table 1). Quantitative real-time PCR (qRT-PCR) was used to detect the relative levels of top ten miRNAs in serum exosomes from 50 patients with EC, corresponding to 30 patients without LN metastasis and 20 patients with LN metastasis, and 50 normal controls. Compared with the control group, the expression level of serum exosomal miR-26a-5p was remarkably reduced in EC patients, especially patients with LN metastasis ($P < 0.001$) (Fig. 1B and Supplementary Fig.1). Consistently, qRT-PCR analyses in 50 normal endometrial tissues, 30 EC tissues without LN metastasis and 20 EC tissues with LN metastasis showed that miR-26a-5p was markedly downregulated in EC tissues with LN metastasis (Fig. 1C). Pearson correlation analyses showed that serum exosomal miR-26a-5p was positively correlated with the expression levels of miR-26a-5p in EC tissue specimens ($r = 0.718$, $P < 0.001$) (Fig. 1D). In addition, we detected miR-26a-5p and exosomal marker protein CD9 expression in 50 EC tissues and 50 normal endometrial tissues paraffin-embedded serial sections using fluorescence in situ hybridization (FISH). The result showed that more colocalized of miR-26a-5p and CD9 signals were observed in overlapping areas in normal endometrial tissues (Supplementary Fig. 2), indicating EC-derived exosomes contained low level of miR-26a-5p.

Next, to identify the correlation between miR-26a-5p level and LN metastasis in EC patients, we also detected miR-26a-5p and LYVE-1 expression using FISH. Compared with the control group, a significantly low level of miR-26a-5p was detected at the primary tumor site in the LN metastasis group (Fig. 1E). Correspondingly, lower miR-26a-5p levels strongly correlated with the increment of peritumoral lymphatic vessel density in sections of EC with LN metastasis specimens, as indicated by the specific lymphatic vessel marker LYVE-1 using Immunofluorescence ($r = -0.6215$, $P < 0.001$) (Fig. 1F). Next, we detected the serum exosomal miR-26a-5p levels between twenty-five paired pre-operative samples and post-operative samples. We found that serum exosomal miR-26a-5p levels in post-operative blood samples were significantly higher than those in pre-operative samples, suggesting serum exosomal miR-26a-5p was related to EC lesions ($P < 0.05$) (Fig. 1G). Receiver operating characteristic (ROC) analysis revealed that serum exosomal miR-26a-5p could discriminate LN metastasis in patients with EC, as measured by the
area under the curve (AUC) (Fig. 1H). Then, analyses of The Cancer Genome Atlas (TCGA) database consistently showed that miR-26a-5p was downregulated in patients with EC (Fig. 1I). And, the expression of miR-26a-5p in tumor tissues was also downregulated than in paracancerous tissues (Fig. 1J). In addition, ROC curves showed the diagnostic value of miR-26a-5p in EC from TCGA data (Fig. 1K). Most importantly, as presented in Table 2, we found LN status and FIGO stage \( (P < 0.05) \) were associated with low level of miR-26a-5p in serum exosomes from EC patients. Collectively, these results indicated that low level of exosomal miR-26a-5p expression was positively associated with LN metastasis in EC.

**EC-secreted exosomes transferred miR-26a-5p to HLEC**

Previous studies have suggested that miRNA can be transferred from cells to cells by exosomes [10]. Thus, we investigated whether miR-26a-5p could be transferred from EC cells to HLEC. Exosomes extracted from the culture medium of EC cell lines (ISK and HEC-1B), and exosomes with a typical membrane-encapsulated particles and 30 - 150 nm in size were confirmed by transmission electron microscopy (TEM) and NanoSight analysis. Western blot revealed that the exosomal protein markers CD9, CD63 and CD81 were positive (Fig. 2A). Furthermore, we found that miR-26a-5p expression was significantly downregulated in EC cells (ISK, AN3CA, KLE, HEC-1A, HEC-1B), and miR-26a-5p was lowly enriched in HEC-1B-secreted exosomes, relative to its cellular content and compared with exosomes derived from non-carcinoma endometrial epithelial cell (EEC) (Fig. 2B and Fig. 2C). We subsequently detect miR-26a-5p in HLEC and found that miR-26a-5p expression was remarkably decreased after incubation with EC cells-secreted exosomes compared with EEC cell-derived exosomes (Fig. 2D). Next, HLEC was incubated with PKH67-labeled (green) exosomes extracted from HEC-1B cell that were transfected with Cy3-labeled (red) miR-26a-5p mimic. Cy3 fluorescence and PKH67 green lipid dye were both observed in the incubated HLEC, while no PKH67 and Cy3 fluorescent signal were observed in the control group, indicating that the HLEC internalized the EC cell-secreted exosomes (Fig. 2E). The same phenomenon was also observed by confocal microscopy (Fig. 2F). qRT-PCR analysis of miR-26a-5p expression in HLEC incubated with exosomes derived from HEC-1B_vector and HEC-1B_mir during different time period (Fig. 2G). These results suggested that cancer cell-secreted miR-26a-5p could be transferred to HLEC through exosomes.

**EC cell-derived exosomal miR-26a-5p promoted lymphangiogenesis in vitro**

Since lymphangiogenesis is a key step for LN metastasis [11], we explored whether downregulating exosomal miR-26a-5p expression could promote lymphangiogenesis in vitro. To investigate the role of exosomal miR-26a-5p in lymphangiogenesis, lentiviral vector overexpressing miR-26a-5p or negative control (NC) was transfected into HEC-1B, and the lentiviral vector silencing miR-26a-5p or NC was transfected to ISK. The tube formation assay and cell migration in HLEC incubated with EC cell-secreted exosomes were analyzed. The EC cell-derived exosomes significantly promoted HLEC tube formation and migration as compared with the control (Fig. 3A). Furthermore, exosomes derived from miR-26a-5p-overexpressing HEC-1B cells (HEC-1B-exo\textsubscript{miR}) dampened the ability to induce HLEC tube formation and migration (Fig. 3B). Conversely, the exosomes derived from miR-26a-5p-silenced ISK cell (ISK-exo\textsubscript{si-miR})
strongly promoted HLEC tube formation and cell migration (Fig. 3C). These results suggested that exosomal miR-26a-5p derived from EC cell induces lymphangiogenesis in vitro.

**Ectopic expression of exosomal miR-26a-5p inhibited EC tumorigenesis and lymphatic metastasis in vivo**

To investigate the suppression of tumorigenicity of exosomal miR-26a-5p in vivo, we used a subcutaneous xenograft model. Mice were inoculated subcutaneously with HEC-1B cells and randomly separated into three groups (n = 5). Each group received intratumoral PBS (control), exosomes secreted by vector-transfected HEC-1B cell (HEC-1B-exo<sub>vector</sub>), or exosomes secreted by miR-26a-5p-transfected HEC-1B cell (HEC-1B-exo<sub>miR</sub>) every 3 days for 3 consecutive weeks. HEC-1B-exo<sub>miR</sub> decreased tumor growth compared with both HEC-1B-exo<sub>vector</sub> group and the control group (Fig. 4A and B). Tumors in HEC-1B-exo<sub>miR</sub> group were of smaller size and weight (Fig. 4C and D) and had lower expression levels of the proliferation marker Ki67 as compared with the HEC-1B-exo<sub>vector</sub> and control groups (Fig. 4E). These results indicated that ectopic expression of exosomal miR-26a-5p could inhibit tumorigenesis.

To further assess the effect of exosomal miR-26a-5p on EC lymphatic metastasis, we established a popliteal lymphatic metastasis model. Luciferase-labeled HEC-1B cell was implanted in the footpads of NOD-SCID mice, which were then randomly divided into three groups (n = 5), followed by intratumoral injection with PBS, HEC-1B-exo<sub>vector</sub>, or HEC-1B-exo<sub>miR</sub> every 3 days. IVIS live in vivo imaging system showed that HEC-1B-exo<sub>miR</sub> significantly inhibited the ability of HEC-1B cell to metastasis to the LN as compared with the control or HEC-1B-exo<sub>vector</sub> groups (Fig. 4F-H). The volume and weight of footpad tumor in the HEC-1B-exo<sub>miR</sub> group were significantly smaller than those in the control or HEC-1B-exo<sub>vector</sub> groups (Fig. 4I and J). When the primary tumors grew to ~50 mm3 after every 3 days for 4 consecutive weeks, we killed the mice and harvested the tumors and the LNs for FISH and IHC analysis. Luciferase immunostaining indicated decreased metastatic LN in the HEC-1B-exo<sub>miR</sub> group, which suggested that ectopic expression of exosomal miR-26a-5p significantly inhibited the metastatic ability of EC cells to LN (Fig. 4K). A significantly lower level of miR-26a-5p was present in some peritumoral lymphatic vessels and tumor cells treated exosomes with HEC-1B-exo<sub>vector</sub> and PBS compared with that with high miR-26a-5p secreted from HEC-1B-exo<sub>miR</sub> (Fig. 4L).

**EC cell-secreted exosomal miR-26a-5p targeted LEF1 to promote lymphangiogenesis**

To explore how miR-26a-5p could regulate lymphangiogenesis, two mRNA target-predicting algorithms (miRDB and TargetScan) were used to predict the potential downstream targets of miR-26a-5p. GeneCards website (http://www.genecards.org) was used to identify relative regulators of endometrial cancer and lymphatic metastasis. Among these candidates, LEF1 was overlapped among all databases (Fig. 5A). To examine whether LEF1 is a target of miR-26a-5p, wild type and mutant type 3′-UTR of LEF1 were respectively cloned into the pMIR-REPORT luciferase plasmid in 293T cell. Notably, the luciferase activities of 3′-UTR of LEF1 was suppressed by miR-26a-5p (Fig. 5B). In addition, ectopic expression of miR-26a-5p in HLEC inhibited the expression of LEF1. Conversely, knocking down of miR-26a-5p resulted
in upregulation of LEF1 (Fig. 5C). CCND1 (β-catenin), LEF1, c-myc, and VEGFA have strong protein-protein interaction. Overexpression of miR-26a-5p in HLEC decreased the levels of β-catenin, LEF1, c-myc, and VEGFA, while restoration of LEF1 expression abrogated these effects (Fig. 5D). Moreover, overexpression of LEF1 rescued the biologic effects related with exosomal miR-26a-5p through tube formation and cell migration assays (Fig. 5E). The downregulated exosomal miR-26a-5p transferred to HLEC decreased the posttranscriptional suppression, activating LEF1/c-myc/VEGFA axis. These results indicated that miR-26a-5p regulated lymphangiogenesis by targeting LEF1 in HLEC.

**Transcription factor EB regulated cellular and exosomal miR-26a-5p in EC**

The decrease in EC cellular and exosomal miR-26a-5p levels accompany the enhancement of lymphangiogenesis in HLEC cell. Growing evidence indicates that transcription factor (TF) plays a vital role in tumor metastasis [12], and regulates multiple miRNAs or protein expression in cancers as well. To determine the potential TFs that are responsible for the downregulation of miR-26a-5p, mRNA sequencing between EEC and EC cells was performed (Fig. 6A). In addition, intersection analysis of the mRNA sequencing results and transcription factors predicted by the JASPAR database (http://jaspar.genereg.net/) showed that three candidates might directly regulate the expression of miR-26a-5p (Fig. 6B). Consistently, analyses of TCGA database showed that transcription factor EB (TFEB) was downregulated in EC (Fig. 6C). As Fig 6D showed that the TFEB prediction of sequence motifs of miR-26a-5p binding sites. The protein and mRNA expression levels of TFEB were also found decreased in HEC-1B and ISK cells as compared with EEC (Fig. 6E and F). Moreover, qRT-PCR analyses showed that the mRNA levels of pre-miR-26a-5p-1 and pre-miR-26a-5p-2 were both decreased in HEC-1B and ISK cells compared with those in EEC (Fig. 6G). Overexpression of TFEB promoted the expression of miR-26a-5p, pre-miR-26a-5p-1, and pre-miR-26a-5p-2 in EC cells, whereas downregulation of TFEB also inhibited the expression (Fig. 6H). Moreover, qRT-PCR analysis showed that exosomal miR-26a-5p level was significantly higher of HEC-1B_{TFEBov} compared with HEC-1B_{TFEBnc} (Fig. 6I). The tube formation and cell migration assay in HLEC incubated with HEC-1B_{TFEBov}-derived exosomes reduced the lymphangiogenesis and cell migration, whereas HEC-1B_{TFEBsi}-derived exosomes enhanced the ability (Fig. 6J).

To determine the interaction effect between TFEB and miR-26a-5p, transwell migration/invasion assay results showed that downexpression of TFEB could promote the migratory and invasive abilities, while restoration of miR-26a-5p expression abrogated these effects. In contrast, overexpression of TFEB significantly inhibited the migratory and invasive abilities (Supplementary Fig. 3 and Fig. 4). Increasing evidence has suggested that the TFEB regulated PI3K/AKT pathways involved in cancer metastasis. To gain further insight into how TFEB suppress EC cells migration and invasion, we found that downexpression of TFEB could increase the phosphorylation levels of PI3K and AKT, while restoration of miR-26a-5p expression abrogated these effects (Fig. 6K). Collectively, these data indicated that TFEB could regulate miR-26a-5p expression and promoted EC cells migratory and invasive abilities through PI3K/AKT activation.
To validate a direct binding of TFEB to the promoter of pre-miR-26a-5p-1 and pre-miR-26a-5p-2, we conducted a chromatin immunoprecipitation (ChIP)-PCR assay using anti-TFEB antibody (Fig. 6L). The results indicated strong enrichment of TFEB in the promoter regions. Furthermore, as Fig 6M showed that Southern blot of pre-miR-26a-5p-1 and pre-miR-26a-5p-2 indicated segments after ChIP assay with antibodies anti-TFEB, respectively. Then, to further demonstrate the regulation of the promoter regions of pre-miR-26a-5p-1 and pre-miR-26a-5p-2 by TFEB, we constructed the promoter-luciferase reporter plasmid systems, and they were co-transfected with TFEB overexpression plasmids into 293T cells. We performed a luciferase reporter assay after transfection. The results showed that TFEB could both significantly increase the activity of pre-miR-26a-5p-1 and pre-miR-26a-5p-2 promoter reporter (Fig. 6N). All these suggested that TFEB could directly bind the specific DNA sequences of pre-miR-26a-5p-1 and pre-miR-26a-5p-2 and influenced the expression of mature miR-26a-5p in EC cells. Taken together, these results further supported our conclusion that TFEB regulated the expression of miR-26a-5p, EC cell-secreted exosomal miR-26a-5p could be transferred to HLEC and promoted lymphangiogenesis by targeting LEF1/c-myc/VEGFA axis (Fig. 7).

Discussion

Recently, many studies have shown that circulating exosomal miRNAs were recognized as promising biomarkers for cancer patients owing to serum exosomal contents altered during cancer progression, as well as their non-invasive diagnostic method and high stability in circulation [13, 14]. Herein, we initially compared the miRNA profiles of serum exosomes derived from 4 patients with EC and 4 healthy donors using miRNA-seq. Subsequently, qRT-PCR was used to detect the relative levels of the top ten miRNAs in serum exosomes from 50 EC patients (30 cases of patients without LN metastasis, 20 cases of patients with LN metastasis) and 50 normal controls. We found that serum exosomal miR-26a-5p was significantly downregulated in the EC patients with LN metastasis compared with the controls. Consistently, miR-26a-5p expression level in 50 EC tissue sections was also lower than that in normal tissue sections. Pearson correlation analyses showed that serum exosomal miR-26a-5p was positively correlated with the level of miR-26a-5p expression in EC tissue specimens. To further verify the serum exosomal miR-26a-5p associated with EC lesions, qRT-PCR analysis showed that serum exosomal miR-26a-5p in post-operative blood samples obviously upregulated after surgical treatment. In addition, ROC curve analysis showed that serum exosomal miR-26a-5p had a good diagnostic potential for EC patients with LN metastasis, indicating that exosomal miR-26a-5p could be utilized for early detection of LN metastasis. Therefore, circulating exosomal miR-26a-5p could serve as a biomarker for early recognition of the LN metastasis in patients with EC.

Regional LN metastasis is the first step of tumor dissemination for a variety of cancers, such as breast cancer, colon, and prostate as well as melanoma [4, 15]. The extent of LN metastasis is a major determining factor for the staging and the prognosis of most malignancies, which often contributes to therapeutic decisions. And it is known that the density of lymphatic vessels is associated with the incidence of LN metastasis and poor prognosis [4, 16]. Cancer-induced lymphangiogenesis plays a crucial part in the metastatic spread of tumor cells [3]. Nonetheless, the molecular mechanisms of
lymphatic metastasis have not well been determined. Previous studies showed that tumor-secreted lymphangiogenic growth factors such as VEGFC and VEGFD could drive the intratumoral lymphangiogenesis [17, 18], and the expression of CCL21 on lymphatic endothelial cell (LEC) could promote tumor cell entry into lymphatic vessels [19]. In addition to molecular mechanism, cell-cell communication in tumor microenvironment that drives lymphangiogenesis has been defined [3]. Exosomal miRNAs have been extensively studied for their role in intercellular communication between tumor cell and tumor microenvironment [20], which results in efficient silencing of mRNAs to regulate the target cell transcriptome [21]. Zeng et al. reported that cancer-derived exosomal miR-25-3p was transferred to vascular endothelial cell to promote vascular permeability and metastatic dissemination [22]. Loss of exosomal antitumor miR-320a derived from cancer-associated fibroblasts cell contributes to hepatocellular carcinoma proliferation and metastasis [23]. Furthermore, downregulation of miR-26a-5p has been reported to be involved in promoting cancer metastasis [24]. However, the role of miR-26a-5p in LEC has not been elucidated. In this study, we found that miR-26a-5p expression was significantly downregulated in EC cells and lowly enriched in EC cell-derived exosome. And we observed Cy3 and PKH67 fluorescence in HLEC incubated with exosomes from HEC-1B cells that were transfected with Cy3-labeled miR-26a-5p. Moreover, the exosomal miR-26a-5p internalized into HLEC targeted LEF1 to promote lymphangiogenesis, thus facilitating lymphatic metastasis. These findings provide mechanistic and translational insights into the signal axis by which exosomal miR-26a-5p promotes EC lymphatic metastasis and that miR-26a-5p may serve as a novel therapeutic target in EC.

LEF1, a member of the T-cell factor (TCF)/LEF1 family, is primarily involved in the canonical Wnt/β-catenin signaling pathway. Zhan et al. reported that upregulation of LEF1 was closely associated with lymphatic metastasis [25]. Consistently, the current study provides evidences that the downregulation of exosomal miR-26a-5p internalized by HLEC cell triggers upregulation of LEF1, contributing to lymphangiogenesis and tumor cell metastasis. And we further confirmed that LEF1 was a novel direct target of miR-26a-5p by luciferase reporter assay. Furthermore, ectopic expression of miR-26a-5p could reduce the mRNA and protein levels of LEF1 in HLEC cell and inhibit tube formation and migration. These findings were further confirmed by the restoration of LEF1 expression. Subsequently, we investigated the possible downstream pathway involved in the miR-26a-5p/LEF1 axis in HLEC. Since the promoter for c-myc contains LEF1 consensus sequences, LEF1 could bind and modulate c-myc transcription [26], which is associated with tumorigenesis and progression of multiple cancers [27-29]. More importantly, c-myc could regulate vascular endothelial growth factor A (VEGFA) to induce lymphangiogenesis and promote lymphatic metastasis [30, 31]. In this study, we demonstrated that the ectopic expression of miR-26a-5p could significantly affect the protein expression of LEF1, c-myc, and VEGFA, while restoration of LEF1 expression abrogated these effects. Therefore, our result indicated that the loss of miR-26a-5p upregulated LEF1 protein expression and subsequent downstream c-myc/VEGFA activation in HLEC cell.

Another key finding of our study was that TFEB could directly bind the promoter regions of pre-miR-26a-5p-1 and pre-26a-5p-2, finally promoting the transcription of mature miR-26a-5p by ChIP-PCR and luciferase activity assay. TFEB belongs to the microphthalmia family of bHLH-leucine zipper molecules. The effect of TFEB on cell proliferation has been extensively investigated. In this study, we demonstrated
that TFEB was downregulated in EC cells and directly regulated the expression of miR-26a-5p, affecting the progression and metastatic ability of EC cells by increasing the phosphorylation of PI3K/AKT.

**Conclusion**

In summary, our findings provided evidence that low level of circulating exosomal miR-26a-5p was associated with LN metastasis in EC patients. Compared with EEC, EC cell-derived exosomes contained low level of miR-26a-5p, and these miR-26a-5p-devoid exosomes transferred into HLEC may induce lymphangiogenesis by targeting LEF1 signaling, which promoted lymphatic metastasis. Our study not only identifies a critical mechanism of exosomal miR-26a-5p-mediated intercellular communication from EC cell to HLEC cell to drive LN metastasis, but also provides a potential non-invasive diagnostic and therapeutic target for EC patients with LN metastasis.

**Abbreviations**

EC: Endometrial cancer, LN: Lymph node, HLEC: Human lymphatic endothelial cell, LEF1: Lymphoid enhancer binding factor 1, TFEB: Transcriptional factor EB, EEC: Endometrial epithelial cell, ISK: Ishikawa, FISH: Fluorescence in situ hybridization, qRT-PCR: Quantitative real-time polymerase chain reaction, ChIP: Chromatin immunoprecipitation, ROC: Receiver operating characteristic, AUC: Receiver operating characteristic, TCGA: The Cancer Genome Atlas, NC: Negative control, LEC: lymphatic endothelial cell, VEGFA: Vascular endothelial growth factor A

**Declarations**

**Acknowledgements**

Not applicable

**Authors’ contributions**

Jing Wang and Xiaodi Gong conceived of the study and its design, performed the experiments and wrote the manuscript. Linlin Yang and Lijuan Li analysed the data and prepared the manuscript. Xiaoyan Gao and Ting Ni helped in animal experiments. Xiaoming Yang, Qiong Fan and Yunxia Cui assisted in data analysis. Xiao Sun and Yudong Wang supervised the overall research, revised manuscript. All authors read and approved the final manuscript. Jing Wang and Xiaodi Gong contributed equally to this work.

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**Availability of data and materials**

The data supporting the findings of this article is included within this article and its supplementary information files.

**Ethics approval and consent to participate**

The study was approved by the Ethics Committee of International Peace Maternity and Child Health Hospital.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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Tables

Table 1 The top ten significantly dysregulated in EC serum exosomes compared with healthy donors’
| miRNA       | chrom  | LogFC | P value  | adj. P   | Regulation |
|------------|--------|-------|----------|----------|------------|
| hsa-miR-183-5p | chr7   | 1.81  | 2.06E-6  | 2.81E-3  | up         |
| hsa-miR-182-5p | chr7   | 1.61  | 6.69E-6  | 4.55E-3  | up         |
| hsa-miR-516b-5p | chr19  | 2.24  | 5.66E-6  | 2.57E-2  | up         |
| hsa-miR-411-3p  | chr14  | -2.87 | 9.24E-5  | 3.14E-2  | down       |
| hsa-miR-873-5p  | chr9   | 5.77  | 1.95E-4  | 4.42E-2  | up         |
| hsa-miR-15a-5p  | chr13  | 1.44  | 1.91E-4  | 4.42E-2  | up         |
| hsa-miR-101-3p  | chr1;chr9 | 0.80  | 4.43E-4  | 4.49E-2  | up         |
| hsa-miR-26a-5p  | chr12;chr3 | -0.97 | 4.21E-4  | 4.49E-2  | down       |
| hsa-miR-21-5p  | chr17  | -0.91 | 4.21E-4  | 4.49E-2  | down       |
| hsa-miR-199a-3p | chr1;chr19 | -1.06 | 3.02E-4  | 4.49E-2  | down       |

Table 2 Correlation of clinical parameters with the expression of serum exosomal miR-26a-5p in patients with EC
| Variable                  | Patients (n) | Fold change     | P value |
|--------------------------|--------------|-----------------|---------|
| **miR-26a-5p**           |              |                 |         |
| Age, y                   |              |                 |         |
| ≥60                      | 21           | 2.625±0.370     | 0.941   |
| <60                      | 29           | 2.595±0.225     |         |
| Grade                    |              |                 |         |
| G1 ~ G2                  | 39           | 2.710±0.216     | 0.216   |
| G3                       | 11           | 2.021±0.444     |         |
| FIGO stage               |              |                 |         |
| I ~ II                   | 27           | 2.307±0.193     | 0.018   |
| III ~ IV                 | 23           | 3.306±0.429     |         |
| Histological type        |              |                 |         |
| Endometrioid             | 37           | 2.665±0.210     | 0.441   |
| Non-endometrioid         | 13           | 2.198±0.600     |         |
| Myometrial invasion      |              |                 |         |
| <1/2                     | 34           | 2.544±0.217     | 0.611   |
| ≥1/2                     | 16           | 2.773±0.449     |         |
| Positive lymph nodes     |              |                 |         |
| No                       | 30           | 2.367±0.206     | 0.022   |
| Yes                      | 20           | 3.432±0.433     |         |
| Lymphovascular space involvement | |                 |         |
| No                       | 29           | 2.740±0.278     | 0.484   |
| Yes                      | 21           | 2.459±0.285     |         |

All data are presented as the means ± SD of three independent experiments.

P ≤ 0.05 was considered significant. The bold type represents P values smaller than 0.05.
Figure 1

Serum exosomal miR-26a-5p is associated with LNs metastasis in EC patients. (A) Heatmap showing the differently expression miRNAs from 4 healthy donors and 4 EC patients. (B) qRT-PCR analysis of miR-26a-5p level in circulating exosomes from healthy donors or EC patients. (C) qRT-PCR analysis of miR-26a-5p expression in normal endometrial tissue and EC tissue with or without LN metastasis. (D) Pearson correlation analysis between miR-26a-5p level in EC tissues and miR-26a-5p level in circulating exosomes from EC patients. (E) Representative FISH images of miR-26a-5p and LYVE-1 expression in paraffin-embedded normal endometrial tissue, EC tissue with or without LN metastasis. (F) Pearson correlation analysis between miR-26a-5p expression and LYVE-1 expression in endometrial tissue. (G) qRT-PCR analysis of serum exosomal miR-26a-5p between pre-operative and post-operative blood samples. (H) ROC curve analysis for evaluating the diagnostic potential of serum exosomal miR-26a-5p for LN metastasis in EC patients. (I) Analysis of miR-26a-5p expression in EC tissue from TCGA data. (J) Analysis of miR-26a-5p expression in EC tumor tissues and paracancerous tissues from TCGA data. (K) ROC curve analysis for evaluating the diagnostic potential of serum exosomal miR-26a-5p for EC from
EC-secreted miR-26a-5p is transferred to human lymphatic endothelial cell. (A) Purified exosomes were identified by Transmission Electron Microscopy, NanoSight, and Western blot analysis. (B) qRT-PCR analysis of basic miR-26a-5p expression in EEC and EC cells. (C) miR-26a-5p level in HEC-1B cell and paired exosomes were detected by qRT-PCR compared with EEC. (D) qRT-PCR analysis of miR-26a-5p expression in HLEC incubated with exosomes derived from EEC, HEC-1B, ISK for 24 h. (E,F) Representative images of Cy3 fluorescence and PKH67 lipid dye in HLEC after adding PKH67-labeled exosomes derived from HEC-1B cells for 48 h. Error bars represent the mean ± SD of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001
experiments. (G) qRT-PCR analysis of miR-26a-5p expression in HLEC incubated with exosomes derived from HEC-1B-exovector and HEC-1B-exomiR. * p < 0.05, ** p < 0.01, *** p < 0.001

Figure 3

Exosomal miR-26a-5p promotes lymphangiogenesis in vitro. (A) Representative images and quantification of tube formation and transwell migration by HLEC treated with PBS, EEC-exo, HEC-exo, or ISK-exo. (B) Representative micrographs and quantification of tube formation and transwell migration in HLEC treated with HEC-1B-exoNC or HEC-1B-exomiR. (C) Representative micrographs and quantification of tube formation and transwell migration in HLEC treated with ISK-exoNC or ISK-exosi-miR. Error bars represent the mean ± SD of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001
Figure 4

Exosomal miR-26a-5p inhibits EC tumorigenesis and lymphatic metastasis in vivo. (A) Representative bioluminescence images and histogram analysis of subcutaneous tumors from NOD-SCID mice treated with PBS, HEC-1B-exovector, or HEC-1B-exomiR (n=5). (B) Representative images of subcutaneous tumors from mice treated with PBS, HEC-1B-exovector, or HEC-1B-exomiR (n=5). (C,D) The measured tumor volumes and weights (n=5). (E) Representative HE and immunohistochemical staining for Ki67 expression. (F) Representative bioluminescence images and histogram analysis of popliteal metastatic LNs from NOD-SCID mice treated with PBS, HEC-1B-exovector, or HEC-1B-exomiR after HEC-1B cells had been inoculated into the footpad (n=5). (G) Representative image of the popliteal LNs metastasis model. (H) Representative images of the popliteal LNs. (I, J) The measured footpad tumor volumes and weights (n=5). (K) IHC staining of luciferase in popliteal LNs from mice treated with the indicated exosomes.
Metastasis-positive LNs were identified by staining for cancer cell-expression luciferase. (L) Staining of miR-26a-5p and LYVE-1 in section of mice footpad tumors. Error bars represent the mean ± SD of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001

Figure 5

EC-secreted exosomal miR-26a-5p targets LEF1 to induce lymphangiogenesis in HLEC. (A) Overlapping results among the prediction of the targets of miR-26a-5p from differential databases. (B) RNA sequence alignment between miR-26a-5p and the 3' UTR of LEF1 (left), and the effect of miR-NC and miR-26a-5p on the activity of the luciferase reporter containing either wild type (WT) or mutant type (MT) were tested by dual-luciferase reporter assay (right). (C) Protein and RNA levels of LEF1 were detected by western blot and qRT-PCR in HLEC transfected with miR-26a-5p mimic/inhibitor or negative control (NC) compared with those treated with the indicated exosomes, respectively. (D) Protein levels of β-catenin, LEF1, c-myc, VEGFA were detected by western blot in HLEC treated with miR-26a-5p mimic or indicated exosomes in the presence of LEF1 overexpression plasmid or NC, respectively. (E) Overexpression of LEF1 rescued the biologic effects related with exosomal miR-26a-5p through tube formation and cell migration assays. Error bars represent the mean ± SD of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001
Figure 6

Transcription factor EB regulates the expression of miR-26a-5p. (A) Heatmap of differential mRNAs between EEC and EC cells after mRNA sequencing. (B) Overlapping results among the differential mRNA from mRNA sequencing and potential directly transcription factors of miR-26a-5p predicted by JASPAR database. (C) Analysis of TFBEB expression in EC tissue from TCGA data. (D) TFBEB prediction of sequence motifs of miR-26a-5p binding sites. (E) Protein level and mRNA level of TFBEB were determined by western blot and qRT-PCR. (F) mRNA level of pre-miR-26a-5p-1 and pre-miR-26a-5p-2 in EC cell compared with EEC cell. (G) qRT-PCR analysis of pre-miR-26a-5p-1 and pre-miR-26a-5p-2 in EC cell compared with EEC cell. (H) TFBEB overexpression/downexpression plasmids were transfected into EC cells, and mRNA levels of miR-26a-5p, pre-miR-26a-5p-1, and pre-miR-26a-5p-2 were detected by qRT-PCR. (I) The mRNA level of miR-26a-5p in HEC-1B-derived exosomes after transfected with TFBEB overexpression plasmid was detected. (J) Representative micrographs and quantification of transwell migration and tube formation in HLEC treated with HEC-1B-derived exosomes after transfected with overexpression/downexpression
plasmids compared with NC-exo. (K) Western blot results showed the phosphorylated PI3K and AKT in HEC-1B cells with the miR-26a-5p mimic/inhibitor after transfected with TFEB downexpression/overexpression plasmids. (L) The relative expression of segments containing TFEB binding sites was detected by qRT-PCR after ChIP assay. (M) Southern blot of indicated segments after ChIP assay with antibodies anti-TFEB. (N) Relative luciferase activity of pre-miR-26a-5p-1 and pre-miR-26a-5p-2 in HEC-1B after transfected with the luciferase reporter containing either wild type (WT) or mutant type (MT). Error bars represent the mean ± SD of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001

Figure 7
Illustrative model showing the mechanism that EC cell-secreted exosomal miR-26a-5p-mediated LEF1 activation in HLEC for promoting lymphangiogenesis and LN metastasis.

**Supplementary Files**

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