Original Paper

Pro-Angiogenic Role of LncRNA HULC in Microvascular Endothelial Cells via Sequestering miR-124

Dexin Yin^a Yezhou Li^a Changgeng Fu^b Ye Feng^c

^aDepartment of Vascular Surgery, China-Japan Union Hospital of Jilin University, Changchun, ^bThe 4th Department of Surgery, DongFang Hospital of Beijing University of Chinese Medicine, Beijing, ^cDepartment of Gastrointestinal Colorectal and Anal Surgery, China-Japan Union Hospital of Jilin University, Changchun, China

Key Words

LncRNA HULC • Atherosclerosis • Angiogenesis • miR-124 • MCL-1 • HMEC-1 cell

Abstract

Background/Aims: HULC is a multifunctional lncRNA that has pro-angiogenic function in various cancers. The present study was designed to see the role of lncRNA HULC in normal endothelial cells angiogenesis. **Methods:** Cell viability, apoptosis, migration, tube formation and expression levels of angiogenesis-related proteins were respectively assessed in human microvascular endothelial HMEC-1 cells after lncRNA HULC was silenced by shRNA transfection. Cross-regulation between lncRNA HULC and miR-124, and between miR-124 and MCL-1 were detected by luciferase reporter assay, sequence analysis, and luciferase reporter assay. **Results:** Silence of lncRNA HULC significantly reduced viability, migration, tube formation and protein levels of VEGFR, EphA2, and eNOS in HMEC-1 cells. Meanwhile, silence of lncRNA HULC induced apoptosis in HMEC-1 cells, as Bcl-2 was down-regulated, Bax was up-regulated, and caspase-3 and -9 were cleaved. miR-124 expression was negatively regulated by lncRNA HULC, and HULC worked as a molecular sponge for miR-124, in having miR-124 exhausted. Besides, MCL-1 was a target gene of miR-124. Rescue assay results showed that the effects of lncRNA HULC silence on HMEC-1 cells growth, migration and angiogenesis were abolished by miR-124 suppression. Similarly, the effects of miR-124 on HMEC-1 cells were abolished by MCL-1 overexpression. Furthermore, MCL-1 activated PI3K/AKT and JAK/STAT signaling pathways. **Conclusion:** These findings suggest a pro-angiogenic role of lncRNA HULC in microvascular endothelial cells. The pro-angiogenic actions of lncRNA HULC may be through sponging miR-124, preventing MCL-1 from degradation by miR-124.

© 2018 The Author(s)
Published by S. Karger AG, Basel

D. Yin and Y. Li contributed equally to this work.

Ye Feng

Department of Gastrointestinal Colorectal and Anal Surgery, China-Japan Union Hospital of Jilin University No.126, Xiantai Street, Changchun 130033 (China)
E-Mail yefeng0152@sohu.com

Introduction

Atherosclerosis is the principal cause of coronary heart disease, ischemic cerebrovascular disease, and peripheral arterial disease. Atherosclerotic plaques narrow the arterial flow lumen leading to ischemic damage and seriously threatening the lives of people. Although the pathogenesis of atherosclerosis is still unclear, it has been widely accepted that dysfunction of endothelial cell induced by stimuli, such as hypoxia and endotoxin, initiates the process of atherosclerosis. Healthy endothelium prevents thrombus formation, leukocyte adhesion, and smooth muscle cell proliferation, whereas dysfunctional endothelium, known as activated endothelium has opposing properties [1]. An improved understanding of endothelial dysfunction may help for development of novel treatment strategies for atherosclerosis.

Long non-coding RNAs (lncRNAs) are a class of transcribed, non-coding RNA molecules (nt), with length longer than 200 nucleotides [2]. Several lncRNAs, like lncRNA NEPON [3], lncRNA MALAT1 [4], and lncRNA GAS5 [5], have been recognized as angiogenesis regulators by controlling of endothelial cells proliferation, tube formation, and migration. Highly up-regulated liver cancer (HULC) is a 500 nt lncRNA. Aberrant expression of lncRNA HULC is closely related with various cancers, including hepatocellular carcinoma [6], colorectal cancer [7], osteosarcoma [8], and esophageal squamous cell carcinoma [9]. lncRNA HULC has been recognized as a cancer-related lncRNA, and its role in angiogenesis was sporadically revealed, since angiogenesis is a common feature of all cancers. Two *in vitro* studies have studied the pro-angiogenic activity of lncRNA HULC in liver cancer cell [10] and glioma cell [11]. However, whether lncRNA HULC also has a function in angiogenesis of normal endothelial cell has not been revealed.

Mechanistically, it is believed that lncRNA contribute to the pathogenesis of diseases via working as a molecular sponge for various miRNAs, which subsequently inhibit miRNA-mediated functions [12]. General information about miRNA involvement in atherosclerosis has been described [13]. Smoking is associated with a high miR-124 expression in whole blood are at increased risk of developing atherosclerosis at an advanced stage as well as in an early phase (subclinical atherosclerosis) [14]. Another *in vivo* investigation evidenced that homocysteine induced the atherosclerosis possibly via promoting the DNA methylation of miR-124 promoter [15]. Additionally, increased expression of miR-124 could induce an up-regulation of atherosclerosis-related markers, like CD206 [14]. These authors demonstrated miR-124 as an atherosclerosis-related miRNA.

In the current study, we set out to explore the function of lncRNA HULC in normal endothelial cells to evaluate the potential role of lncRNA HULC in atherosclerosis. Then, we focused on the correlation between lncRNA HULC and miR-124, to reveal one of the underlying mechanisms of lncRNA HULC's action in endothelial cells angiogenesis.

Materials and Methods

Microvascular endothelial cells culture

Human microvascular endothelial cell line HMEC-1 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in a culture system using Dulbecco's Modified Eagle's Medium (DMEM, GIBCOL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCOL) and were maintained at 37°C in a humidified incubator (with 5% CO₂ and 95% air).

qRT-PCR analysis

To test the RNA levels of lncRNA HULC and MCL-1, total RNA in HMEC-1 cells was extracted by using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA). 5 µg of total RNA were subjected to reverse transcription by using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). qRT-PCR was conducted on the ABI PRISM 7500 Real-time PCR System (Applied Biosystems, Foster City,

CA, USA) by using FastStart Universal SYBR Green Master (Roche). GAPDH served as an internal control in this procedure. To test the RNA levels of miR-372, miR-186, miR-9, miR-122, miR-195, miR-488 and miR-124, the miRNAs in HMEC-1 cells were isolated by miRNeasy Mini Kit (Qiagen, Shenzhen, China). Reverse transcription was done in 5 µg of total miRNAs by using the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems). Taqman Universal Master Mix II with the TaqMan MicroRNA Assay (Applied Biosystems) was used for qRT-PCR. The RNA levels of miRNAs were normalized to U6. Fold changes were calculated by relative quantification ($2^{-\Delta\Delta Ct}$) method.

Cell transfection

LncRNA HULC was silenced by shRNA transfection. shRNA specific for human LncRNA HULC was ligated into the pGPU6 plasmid which was purchased from Genepharma (Shanghai, China). A non-targeting sequence was transfected as a blank control. The specific mimic, inhibitor and shRNA controls for hsa-miR-124 were all from Genepharma, and were transfected into cells. A 400-bp fragment of MCL-1 was ligated into pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA), and pcDNA3.1 plasmid was used as its blank control. For MCL-1 silencing, shRNA specific for MCL-1 was transfected into pGPU6 plasmid (Genepharma). A non-targeting sequence was used as its blank control. All transfections were performed by using lipofectamine 3000 reagent (Life Technologies Corporation) under antibiotic-free conditions. At 48 h of transfection, the culture medium was replaced with fresh medium containing 0.5 mg/ml G418 (Sigma-Aldrich, St Louis, MO, USA). The G418-resistant cells were selected after four weeks of culturing.

Cell viability assay

Trypan blue (Beyotime, Shanghai, China) was used to detect the viability of HMEC-1 cells. After the indicated transfection, HMEC-1 cells were transfected into 96-well plates with a density of 5×10^4 cells/well, after which the plates were maintained at 37°C for 7 days. At the indicated time periods, cells were collected by trypsin/EDTA and centrifugation. Cells were stained by trypan blue solution at room temperature for 3 min. Living cells were counted microscopically. Viability was calculated as % cell viability = unstained cells (living cells)/total cells $\times 100\%$.

Apoptosis assay

Cell apoptosis was analyzed by using Annexin V Apoptosis Detection kit (BD Biosciences, San Jose, CA, USA). The transfected HMEC-1 cells (5×10^4 cells/sample) were double stained with 10 µl FITC Annexin V and 10 µl PI. After 30 min incubation in the dark over ice, the samples were analyzed under the flow cytometry (FACScan®; BD Biosciences) to distinguish early apoptotic cells (FITC-positive and PI-negative) from death cells and apoptotic cells (FITC-positive and PI-positive). Quantification of the apoptotic cell rate was analyzed by FlowJo software (Tree Star, San Carlos, California, USA).

Migration assay

Cell migration was determined as previously described [16] by using a Transwell chamber (Costar, High Wycombe, UK) with an 8-µm pore filter. The transfected HMEC-1 cells (5×10^4 cells/well) were seeded in the serum-free medium and were placed in the upper side of chamber. The lower chamber was filled with the complete medium (i.e., DMEM medium with 10% FBS). After 48 h of incubation at 37°C, non-traversed cells in the upper side were wiped away carefully by cotton swabs. The cells in lower side were stained with crystal violet and were counted microscopically. Relative cell migration was calculated based on the traversed cell numbers from five randomly selected fields, and was respect to the following formula: relative migration (%) = (cell number in experimental group)/(cell number in control) $\times 100\%$.

Tube formation assay

A 6-well plate was pre-coated with matrigel (BD Biosciences) (1.8 ml/well). After polymerization of matrigel at 37°C for 1 h, HMEC-1 cells were seeded in each well with a density of 1×10^5 cells/well. After 24 h of incubation in DMEM supplemented with 2% FBS, numbers of tube-like cell were counted under phase-contrast microscopy.

Reporter vector construct and luciferase reporter assay

The genomic DNA fragment of MCL-1 which contains the predicted miR-124 binding site was amplified by PCR and cloned into pmirGLO Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). The predicted binding site in MCL-1 was replaced by a non-targeting sequence to form a control reporter vector. The wild-type (wt) and the mutated-type (mt) of vectors were respectively co-transfected with miR-124 mimic by using lipofectamine 3000 reagent (Life Technologies Corporation). After transfection, Dual-Luciferase Reporter Assay System (Promega) was used for testing the luciferase activity.

ELISA

After the indicated transfection, cell culture supernatant of each sample was collected and centrifuged at 1000 *g*, 4°C for 15 min. The concentrations of VEGF in the culture supernatant were tested by using Human VEGF ELISA KIT (CSB-E11718h, Cusabio, Wuhan, China).

RNA immunoprecipitation (RIP)

wt or mt of HULC was inserted into pcDNA3.1-MS2 plasmid (Addgene, Cambridge, MA, USA) and was co-transfected with pMS2-GFP (Addgene) and pcDNA3.1-MS2, pcDNA3.1-HULC or pcDNA3.1-HULC-mt-MS2. Then, the cells were lysed by lysis buffer (25 mmol/l Tris-HCl (pH 7.5), 150 mmol/l KCl, 2 mmol/l EDTA, 0.5% NP40, 1 mmol/l NaF, 1 mmol/l DTT, 100 U/ml RNase inhibitor, and 50% glycerol) containing protease inhibitor. The cell lysate was incubated with magnetic beads and GFP antibody (ab10556, Abcam, Cambridge, MA) by using Magna RIP™ RNA-Binding Protein Immunoprecipitation kit (Millipore, Bedford, MA). IgG antibody (ab200699, Abcam) was used as a blank control. After incubation at 4°C for 6 h, the samples were centrifugated at 800 *g*, 4°C for 10 s. Subsequently, the co-immunoprecipitated RNA were measured by qRT-PCR. For anti-AGO2 RNA IP, cells were transfected with miR-con or miR-124 mimic. anti-AGO2 antibody (ab186733, Abcam) was used in this procedure.

Western blot

HMEC-1 cells were lysed by 1% Triton X-100 and 1 mM PMSF (pH 7.4) over ice for 30 min for protein extraction. Purity and concentration of protein in the supernatant of the whole-cell extracts were qualified by the BCA™ Protein Assay Kit (Bio-Rad, Richmond, CA, USA). Protein (0.1 mg) was separated by SDS-PAGE and was transferred onto a PVDF membrane. The membranes were blocked in 5% non-fat dry milk/0.05% Tween for 1 h at room temperature, and then the blots were probed by primary antibodies overnight at 4°C for the detection of VEGF (orb30395, R&D, Minneapolis, MN, USA), VEGFR2 (orb186489), CD144 (orb375049), eNOS (orb29014), Bcl-2 (orb10173), Bax (orb378567), caspase-3 (sc-271759, Santa Cruz Biotechnology, Santa Cruz, CA, USA), GAPDH (sc-47724), caspase-9 (ab25758, Abcam, Cambridge, MA, USA), p-PI3K (ab109550), PI3K (ab191606), p-AKT (ab38449), AKT (ab8805), p-Jak1 (ab215338), Jak1 (ab47435), p-Tyr (ab3209), Tyk2 (ab223733), p-Stat1 (ab30645), Stat1 (ab31369), p-Stat2 (ab53132), and Stat2 (ab12367). The membranes were then incubated with the secondary antibodies for 1 h at room temperature. After rinsing, the positive bands were visualized by chemiluminescence and autoradiography using X-ray film (Applygen Technologies Inc., Beijing, China). Intensity of bands was quantified using Image software (Bio-Rad, CA, USA).

Statistical analysis

Data were expressed as mean ± SD from three independent experiments. Statistical analysis was performed by SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA). Statistical differences between two groups were analyzed by the ANOVA with a Duncan post-hoc procedure. *p*-values lower than 0.05 were considered as significant differences.

Results

Capillary structure formation of HMEC-1 cells

HMEC-1 cells were seeded in matrigel-coated plates for 1-24 h. As a result, tube formation was increased with the prolonging of time (Fig. 1A). The angiogenesis of HMEC-1 cells was further confirmed by detection of angiogenesis-related proteins. ELISA assay results showed that VEGF concentrations in the culture supernatant were significantly increased with the prolonging of time ($p < 0.05$, Fig. 1B). Meanwhile, Western blotting results showed that protein levels of VEGF, VEGFR2, CD144 and eNOS were all highly expressed with the prolonging of time ($p < 0.05$, Fig. 1C).

Silence of lncRNA HULC represses HMEC-1 cells viability, migration and capillary structure formation

The expression changes of lncRNA HULC were monitored with the prolonging of time. qRT-PCR data in Fig. 2A showed that lncRNA HULC expression was significantly increased with the time increased ($p < 0.05$). Next, two sequences of shRNAs specific for lncRNA HULC were respectively transfected into HMEC-1 cells to knock down the expression of lncRNA HULC in cell. qRT-PCR analytical results indicated that sh-HULC #1 ($p < 0.05$) and sh-HULC #2 ($p < 0.001$) transfection significantly reduced the RNA levels of lncRNA HULC when compared to the sh-NC group (Fig. 2B). Considering that sh-HULC #2 resulted in a lower level of lncRNA HULC than sh-HULC #1, sh-HULC #2 was selected for use in the following shRNA transfection. Viability of cells was significantly reduced in sh-HULC compared to sh-NC at day 3 ($p < 0.05$) and day 4 ($p < 0.001$) post transfection (Fig. 2C). Protein expression of Bcl-2 was down-regulated, Bax was up-regulated, and caspase-3 and -9 were cleaved after sh-HULC transfection (Fig. 2D). More importantly, cell migration ($p < 0.01$) and tube-like

Fig. 1. Capillary structure formation of HMEC-1 cells. (A) HMEC-1 cells were seeded in matrigel-coated plates for 1-24 h. In the indicated time period, tube formation was assessed. (B) Concentrations of VEGF in culture supernatant were measured by ELISA. (C) Protein levels of angiogenesis-related factors were measured by Western blot analysis. Data represented as mean $\pm$ SD ($n = 3$). Different lowercase letters above the columns indicate that the mean values of different groups are significantly different ($p < 0.05$, ANOVA).

Fig. 2. Silence of lncRNA HULC represses HMEC-1 cells viability, migration and capillary structure formation. (A) Expression changes of lncRNA HULC with the prolonging of the time were determined by qRT-PCR. (B) Two sequences (#1 and #2) of shRNAs specific for lncRNA HULC were respectively transfected into HMEC-1 cells. A non-targeting shRNA (sh-NC) was transfected as a blank control. The RNA levels of HULC were monitored by qRT-PCR post-transfection. (C) Viability of HMEC-1 cells was monitored by Trypan blue staining at day 1 to day 4 post-transfection. (D) Protein levels of apoptosis-related factors were measured by Western blot analysis. (E) Relative migration was detected by using a Transwell chamber. (F) Tube formation capacity was detected by counting cells in matrigel coated plates. Concentration of VEGF in culture supernatant were measured by ELISA. Expression levels of angiogenesis-related proteins were measured by Western blotting. Data

represented as mean $\pm$ SD (n = 3). Different lowercase letters above the columns indicate that the mean values of different groups are significantly different (p < 0.05, ANOVA). * p < 0.05, ** p < 0.01, and *** p < 0.001 compared to sh-NC group (ANOVA).

cell rate (p < 0.001) were repressed in sh-HULC group when compared to the sh-NC group (Fig. 2E and 2F). The reduced formation of capillary structure was confirmed in Fig. 2G and 2H, that the concentration of VEGF in culture supernatant (p < 0.001) and the protein levels of VEGF, VEGFR2, CD144 and eNOS were all suppressed by sh-HULC.

Fig. 4. Silencing of lncRNA HULC represses HMEC-1 cells viability, migration and capillary structure formation via sponging miR-124. (A) HMEC-1 cells were transfected with sh-HULC or co-transfection with sh-HULC and anti-miR-124. sh-NC and anti-miR (a scrambled miRNA) were transfected as blank controls for sh-HULC and anti-miR-124. Cell viability was monitored by Trypan blue staining at day 1 to day 4 post-transfection. (B) Apoptotic cell rate was determined by flow cytometry. (C) Protein levels of apoptosis-related factors were measured by Western blot analysis. (D) Relative migration was detected by using a Transwell chamber. (E) Tube formation capacity was detected by culturing cells in matrigel pre-coated plates. (F) Expression levels of angiogenesis-related proteins were measured by Western blotting. Data represented as mean $\pm$ SD (n = 3). * $p < 0.05$ and ** $p < 0.01$ compared to the indicated group (ANOVA).

regulated by lncRNA HULC. Considering that miR-124 has been previously reported as an anti-angiogenic regulator [17], miR-124 was selected for further investigation. Next, the expression changes of lncRNA HULC in response to miR-124 dysregulation were tested. Data in Fig. 3B showed that lncRNA HULC was also negatively regulated by miR-124 ($p < 0.05$). Sequence analytical results in Fig. 3C showed that, there existed three sites in lncRNA HULC which could directly bind with miR-124. To further explore the regulatory relationship between lncRNA HULC and miR-124, RNA IP was performed. Fig. 3D showed that the relative

enrichment to MS2 was much higher in MS2-HULC group than the MS2 group ($p < 0.01$). Additionally, endogenous lncRNA HULC pull-down by AGO2 was significantly enriched in miR-124 group as compared to the miR-con group ($p < 0.001$, Fig. 3E). These findings suggested that lncRNA HULC could sponge miR-124, having miR-124 exhausted by binding effects.

Silence of lncRNA HULC represses HMEC-1 cells viability, migration and capillary structure formation via sponging miR-124

Next, HMEC-1 cells were transfected with sh-HULC or co-transfection with sh-HULC plus anti-miR-124, to see whether miR-124 was involved in the functions of lncRNA HULC in HMEC-1 cells. Fig. 4A displayed that sh-HULC-induced the impairment of cell viability was alleviated by anti-miR-124. sh-HULC-induced increase of apoptotic cell rate was down-regulation of Bcl-2, up-regulation of Bax, and cleavage of caspase-3 and -9 were all alleviated by anti-miR-124 (Fig. 4B and 4C). As expected, sh-HULC-induced inhibition of migration and tube-like cell rate (both $p < 0.05$), and down-regulations of angiogenesis-related proteins were also recovered to the control baseline when anti-miR-124 was co-transfected (Fig. 4D-4F).

MCL-1 is a target gene of miR-124

It has been reported that miR-124 influenced apoptosis regulation of AKT/mTOR/MCL-1 survival pathway [18]. Here, we found that the mRNA ($p < 0.05$) and protein levels of MCL-1 was down-regulated by miR-124 mimic transfection, while was up-regulated by anti-miR-124 transfection (Fig. 5A and 5B). Sequence analytical results in Fig. 5C showed that, MCL-1 possessed four sites which could directly bind with miR-124. To analyze whether MCL-1 could directly bind with miR-124, the predicted binding site in MCL-1 (GTGAACAC) was selected for use in luciferase reporter assay. As results shown in Fig. 5D, the luciferase activity was significantly reduced by co-transfection with MCL-1-wt and miR-124 mimic ($p < 0.05$). Meanwhile, the luciferase activity was significantly increased by co-transfection with MCL-1-mt, miR-124 mimic and pc-HULC ($p < 0.05$). No significant changes were observed in luciferase activity after co-transfection with MCL-1-mt and miR-124 mimic, as well as co-transfection with MCL-1-mt, miR-124 mimic and pc-HULC. These data indicated that MCL-1 was a target gene for miR-124. Next, we performed Western blot analysis for testing the regulatory relationship between lncRNA HULC, miR-124 and MCL-1. Fig. 5E showed that protein level of MCL-1 was up-regulated in pc-HULC group and was down-regulated in sh-HULC group as compared to the corresponding controls respectively. Fig. 5F showed that protein level of MCL-1 was remarkably up-regulated in sh-HULC+anti-miR-124 group as compared to anti-HULC+anti-miR group. These data together with the above mentioned findings implied an interesting regulation between lncRNA HULC, miR-124 and MCL-1, that lncRNA HULC acted as a molecular sponge for miR-124, preventing MCL-1 from degradation by miR-124.

lncRNA HULC represses HMEC-1 cells viability, migration and capillary structure formation via targeting MCL-1

The expression changes of MCL-1 in HMEC-1 cells with the prolonging of the time were determined by performing qRT-PCR. Data in Fig. 6A showed that mRNA level of MCL-1 was significantly up-regulated with the time increased ($p < 0.05$). Next, to test the involvement of MCL-1 in miR-124-mediated functions in HMEC-1 cells, miR-124 mimic and/or pc-MCL-1 were transfected into cell. Viability of HMEC-1 cells was significantly reduced by miR-124 mimic at day 4 post-transfection ($p < 0.01$, Fig. 6B). miR-124 mimic transfection resulted in a significant increase in apoptotic cell rate ($p < 0.001$, Fig. 6C), a down-regulation of Bcl-2, an up-regulation of Bax, and cleavages of caspase-3 and -9 (Fig. 6D). Additionally, relative migration ($p < 0.05$, Fig. 6E), tube-like cell rate ($p < 0.01$, Fig. 6F), and expression levels of

angiogenesis-related proteins (Fig. 6G and 6H) were all repressed by miR-124 mimic. More importantly, these above mentioned functions of miR-124 mimic on HMEC-1 cells were all abolished by pc-MCL-1 transfection.

MCL-1 activates PI3K/AKT and JAK/STAT signaling pathways

For further explore the underlying mechanisms of which MCL-1 promoted HMEC-1 cell angiogenesis, the expression changes of core proteins in PI3K/AKT and JAK/STAT signaling pathways were determined. As shown in Fig. 7A and 7B, sh-MCL-1 transfection reduced

Fig. 5. MCL-1 is a target gene of miR-124. (A) The mRNA and (B) protein levels of MCL-1 were determined by qRT-PCR and Western blot after HMEC-1 cells were transfected with miR-124 (mimic) or anti-miR-124 (inhibitor). miR-con and anti-miR, two scrambled miRNAs, were transfected as blank controls for miR-124 and anti-miR-124. (C) Sequence analysis of the targeting relationship between miR-124 and MCL-1. The red, green, blue and yellow lines represented the pairing sequences. (D) The third predicted binding site in MCL-1 (the sequence colored blue below) was selected to detect whether MCL-1 could bind with miR-124 by using luciferase reporter assay. The protein levels of MCL-1 were measured by Western blot in (E) HMEC-1 cells after transfection with pc-HULC or sh-HULC, as well as in (F) HMEC-1 cells after co-transfection with anti-HULC and anti-miR-124. Data represented as mean $\pm$ SD (n = 3). * p < 0.05 compared to the indicated group (ANOVA).

Fig. 3. miR-124 represses HMEC-1 cells viability, migration and capillary structure formation via targeting MCL-1. (A) The mRNA levels of MCL-1 in HMEC-1 cells were determined by qRT-PCR with the prolonging culture time. (B) HMEC-1 cells were transfected with miR-124 or co-transfection with miR-124 plus pc-MCL-1 (a MCL-1 expression vector). miR-con and pcNC (an empty plasmid) were transfected as blank controls for miR-124 and pc-MCL-1. Cell viability was monitored by Trypan blue staining at day 1 to day 4 post-transfection. (C) Apoptotic cell rate was determined by flow cytometry. (D) Protein levels of apoptosis-related factors were measured by Western blot analysis. (E) Relative migration was detected by using Transwell chamber. (F) Tube formation capacity was detected by culturing cells in matrigel pre-coated plates. (G) Concentrations of VEGF in culture supernatant were measured by ELISA. (H) Expression levels of angiogenesis-related proteins were measured by Western blotting. Data represented as mean $\pm$ SD (n = 3). Different lowercase letters above the columns indicate that the mean values of different groups are significantly different (p < 0.05, ANOVA). * p < 0.05, ** p < 0.01, and *** p < 0.001 compared to the indicated group (ANOVA).

Fig. 7. MCL-1 activates PI3K/AKT and JAK/STAT signaling pathways. HMEC-1 cells were transfected with pc-MCL-1 or sh-MCL-1 (shRNA specific for MCL-1). pcNC and shNC (non-targeting shRNA) were transfected as blank controls for pc-MCL-1 and sh-MCL-1. The expression levels of core proteins in PI3K/AKT pathway were detected by Western blotting. (B) The expression levels of core proteins in JAK/STAT pathway were detected by Western blotting.

phosphorylation levels of PI3K, AKT, Jak1, Tyk2, Stat1, and Stat2, while pc-MCL-1 transfection increased the phosphorylation of these proteins. The total levels of these proteins were unaffected either by pc-MCL-1 or by sh-MCL-1.

Discussion

Recently, a growing number of lncRNAs interfering with the progress of atherosclerosis were identified and characterized in the atherogenic cells like smooth muscle cell, endothelial cell and macrophage [19]. In the present study, we identified lncRNA HULC also as an atherosclerosis-related lncRNA, which was unstudied previously. Silence of lncRNA HULC repressed the proliferation, migration and tube formation, and promoted apoptosis. Mechanistically, lncRNA HULC exerted “sponge-like” effect on miR-124, preventing MCL-1 (a target gene of miR-124) from degradation by miR-124. Therefore, MCL-1 showed accelerating effects on the activation of PI3K/AKT and JAK/STAT signaling pathways in HMEC-1 cells.

Endothelial dysfunction is a fundamental event in the process of atherosclerosis. Endothelial cells switch from a quiescent phenotype toward one that involves the host defense response, during which eNOS switches to generate ROS in appropriate circumstances as a part of endothelial dysfunction [1]. VEGF is a well-known angiogenic growth factor that is released by virtue of its ability to accumulate endothelial cells and to induce angiogenesis and neovasculation [20, 21]. CD144 is a transmembrane protein involved in endothelial cell-cell adhesion [22]. In the present study, we demonstrated that silence of lncRNA HULC decreased the tube formation capacity of HMEC-1 cells, and reduced the expression of VEGF, VEGFR2 (a receptor of VEGF), CD144 and eNOS, implying lncRNA HULC might contribute to endothelial dysfunction and angiogenesis. As the angiogenesis continues, endothelial cells migration and proliferation lead to the more advanced lesions of atherosclerosis. We additionally demonstrated that silence of lncRNA HULC reduced HMEC-1 cells growth and migration. This observation suggested that lncRNA HULC promoted the progression of atherosclerosis via controlling endothelial cells influx and proliferation.

HULC is a multifunctional lncRNA that recent works mainly focused on its role in

various cancers. Angiogenesis is a common feature of all cancers, thus it is rational that investigators have begun to concern about the role of lncRNA HULC in normal endothelial cells angiogenesis. Increased expression of lncRNA HULC in glioma cells created a bridge between VEGF and VEGFR, as well as between angiogenesis and invasion, which contributed to cancer malignancy [11]. An *in vitro* investigation also evidenced that lncRNA HULC was capable of promoting tumor angiogenesis in hepatoma cells [10]. However, the present work for the first time discovered that lncRNA HULC also exerted pro-angiogenic functions on normal endothelial cells.

It has been well-accepted that lncRNA function to the regulation of gene expression via acting as endogenous sponges for various miRNAs [12, 23]. lncRNA HULC could post-transcriptionally sponge miR-186 [24], miR-122 [8], miR-372/miR-373 [25], and miR-124 [26], preventing target genes from degradation by these miRNAs, and thereby participating in a wide range of cellular process, like cell proliferation, differentiation, migration, invasion, apoptosis and inflammatory response. Herein, we found that lncRNA HULC was a molecular sponge for miR-124, that lncRNA HULC sequestered miR-124, preventing miR-124 from binding with miR-124. The role of miR-124 observed in this study is in consistency with previous studies demonstrating miR-124 as an atherosclerosis-related miRNA [14, 15].

MCL-1 is a unique member of Bcl-2 family that is different from other family members (*i.e.*, Bcl-2, Bcl-xL, Bcl-w and Bfl-1A1), as it holds a considerably long C-terminal domain [27]. MCL-1 has been shown to be widely expressed in many types of cells, including endothelial cells in a wide range of tissues, such as prostate, breast, endometrium, skin, dermis, stomach, etc. [28]. MCL-1 exhibited oncogenic functions in colorectal cancer cells by inhibition of apoptosis and enhancing angiogenesis [29]. In the present study, the pro-angiogenic effects of MCL-1 on HMEC-1 cells were observed, and our data suggested that MCL-1 might be one of the executors for lncRNA HULC, bringing lncRNA HULC into a significant role in angiogenesis.

Furthermore, various studies suggest an essential role of PI3K/AKT signaling pathway in the survival and angiogenesis of endothelial cells. Activation of AKT activates eNOS, increases the endothelial NO synthesis, which promotes endothelial cells survival [31] and migration [32], and leads to a pro-angiogenic effect [33]. In addition to PI3K/AKT, JAK/STAT signaling pathway is also essential in mediating the proliferation of vascular endothelial cells [34], which evidences its importance in modulating angiogenesis. In the current study, both PI3K/AKT and JAK/STAT signaling pathways were shown to be activated by MCL-1 overexpression, while were blocked by MCL-1 suppression. These findings suggest PI3K/AKT and JAK/STAT signaling pathways might be involved in the pro-angiogenic functions of MCL-1 in HMEC-1 cells.

Conclusion

Our study and the findings in this study demonstrated a promoting role of lncRNA HULC in endothelial cells angiogenesis. One of the possible mechanisms involved in the pro-angiogenic function is that lncRNA HULC works as a molecular sponge for miR-124, preventing MCL-1 (the target gene of miR-124) from degradation by miR-124. More effort is required to further check the pro-angiogenic role of lncRNA HULC *in vivo* and in other types of endothelial cells, especially the primary cells.

Disclosure Statement

The authors declare that they have no competing interests.

References

- 1 Deanfield JE, Halcox JP, Rabelink TJ: Endothelial function and dysfunction: testing and clinical relevance. *Circulation* 2007;115:1285-1295.
- 2 Mattick JS, Rinn JL: Discovery and annotation of long noncoding RNAs. *Nat Struct Mol Biol* 2015;22:5-7.
- 3 Tang YY, Wo LK, Chai H: [Effects of noncoding RNA NRON gene regulation on human umbilical vein endothelial cells functions]. *Zhonghua Xin Xue Guan Bing Za Zhi* 2013;41:245-250.
- 4 Michalik KM, You X, Manavski Y, Doddaballapur A, Zornig M, Braun T, John D, Ponomareva Y, Chen W, Uchida S, Boon RA, Dimmeler S: Long noncoding RNA MALAT1 regulates endothelial cell function and vessel growth. *Circ Res* 2014;114:1389-1397.
- 5 Chen L, Yang W, Guo Y, Chen W, Zheng P, Zeng J, Tong W: Exosomal lncRNA GAS5 regulates the apoptosis of macrophages and vascular endothelial cells in atherosclerosis. 2017;12:e0185406.
- 6 Sonohara F, Inokawa Y, Hayashi M, Yamada S, Sugimoto H, Fujii T, Koderia Y, Nomoto S: Long Non-Coding RNA HULC and MALAT1 Following the Curative Resection of Hepatocellular Carcinoma. *Sci Rep* 2017;7:16142.
- 7 Shaker OG, Senousy MA, Elbaz EM: Association of rs6983267 at 8q24, HULC rs1042811 polymorphisms and serum lncRNAs CCAT2 and HULC with colorectal cancer in Egyptian patients. *Sci Rep* 2017;7:16246.
- 8 Kong D, Wang Y: Knockdown of lncRNA HULC inhibits proliferation, migration, invasion and promotes apoptosis by sponging miR-122 in osteosarcoma. 2018;119:1050-1057.
- 9 Jiang L, Wang W, Li G, Sun C, Ren Z, Sheng H, Gao H, Wang C, Yu H: High expression of lncRNA HULC is associated with chemotherapy resistance and poor prognosis in esophageal squamous cell carcinoma. *Cancer Chemother Pharmacol* 2016;78:333-339.
- 10 Lu Z, Xiao Z, Liu F, Cui M, Li W, Yang Z, Li J, Ye L, Zhang Y: lncRNA HULC promotes tumor angiogenesis in liver cancer by up-regulating sphingosine kinase 1 (SPHK1). *Oncotarget* 2016;7:241-254.
- 11 Zhu Y, Zhang X, Qi L, Cai Y, Yang P, Xuan G, Jiang Y: lncRNA HULC silencing suppresses angiogenesis by regulating ESM-1 via the PI3K/Akt/mTOR signaling pathway in human gliomas. *Oncotarget* 2016;7:14429-14440.
- 12 Bayoumi AS, Sayed A, Broskova Z, Teoh J, Zhang J, Chen H, Tang YL, Kim IM: Crosstalk between Long Noncoding RNAs and MicroRNAs in health and disease. *Int J Mol Sci* 2016;17:356.
- 13 Volny O, Kasickova L, Coufalova M, Coufalova P, Novak J: microRNAs in Cerebrovascular Disease. *Adv Exp Med Biol* 2015;888:155-190.
- 14 de Ronde MWJ, Kok MG, Verberland P, van den Bossche J, Neele AE, Halliani A, van der Made I, de Winther MPJ, Meijers JC, Greemink EE, Pinto-Sietsma SJ: High miR-124-3p expression identifies smoking individuals susceptible to atherosclerosis. *Atherosclerosis* 2017;263:377-384.
- 15 Zhao L, Jiao Y, Cao G, Kong FQ, Liu XM, Yang XL, Jiang YD: [The effect of miR-124 on homocysteine-induced atherosclerosis via promoter region DNA methylation in ApoE(-/-) mice]. *Sheng Li Xue Bao* 2015;66:107-114.
- 16 Lian Y, Cai Y, Zhang Y, Huang S, Feng W, Gu X: Combretastatin A4 Regulates Proliferation, Migration, Invasion and Apoptosis of Thyroid Cancer Cells via PI3K/Akt Signaling Pathway. *Med Sci Monit* 2016;22:4913-4917.
- 17 Zhang Z, Chen C, Li C, Wang L, Qian X, Jiang C, Liu X, Wang X, Li H, Kang C, Jiang T, Liu LZ, You Y, Liu N, Jiang BH: lncRNA HULC governs glioma growth and angiogenesis and enhances chemosensitivity by targeting R-Ras and N-Ras. *Neuro Oncol* 2014;16:1341-1353.
- 18 Kim J, Jeong D, Nam J, Aung TN, Gim JA, Park KU, Kim SW: MicroRNA-124 regulates glucocorticoid sensitivity by targeting phosphodiesterase 4B in diffuse large B cell lymphoma. *Gene* 2015;558:173-180.
- 19 Jian L, Jian D, Chen Q, Zhang L: Long Noncoding RNAs in Atherosclerosis. *J Atheroscler Thromb* 2016;23:376-384.
- 20 Lobb RR, Key ME, Alderman EM, Fett JW: Partial purification and characterization of a vascular permeability factor secreted by a human colon adenocarcinoma cell line. *Int J Cancer* 1985;36:473-478.
- 21 Springer ML, Chen AS, Kraft PE, Bednarski M, Blau HM: VEGF gene delivery to muscle: potential role for vasculogenesis in adults. *Mol Cell* 1998;2:549-558.
- 22 Lampugnani MG, Corada M, Caveda L, Breviario F, Ayalon O, Geiger B, Dejana E: The molecular organization of endothelial cell to cell junctions: differential association of plakoglobin, beta-catenin, and alpha-catenin with vascular endothelial cadherin (VE-cadherin). *J Cell Biol* 1995;129:203-217.

- 23 Cao MX, Jiang YP, Tang YL, Liang XH: The crosstalk between lncRNA and microRNA in cancer metastasis: orchestrating the epithelial-mesenchymal plasticity. *Oncotarget* 2017;8:12472-12483.
- 24 Wang Y, Chen F, Zhao M, Yang Z, Li J, Zhang S, Zhang W, Ye L, Zhang X: The long noncoding RNA HULC promotes liver cancer by increasing the expression of the HMGA2 oncogene via sequestration of the microRNA-186. *J Biol Chem* 2017;292:15395-15407.
- 25 Wang WT, Ye H, Wei PP, Han BW, He B, Chen ZH, Chen YQ: LncRNAs H19 and HULC, activated by oxidative stress, promote cell migration and invasion in cholangiocarcinoma through a ceRNA manner. *J Hematol Oncol* 2016;9:117.
- 26 Ma Y, Huang D, Yang F, Tian M, Wang Y, Shen D, Wang Q, Chen Q, Zhang L: Long Noncoding RNA Highly Upregulated in Liver Cancer Regulates the Tumor Necrosis Factor-alpha-Induced Apoptosis in Human Vascular Endothelial Cells. *DNA Cell Biol* 2016;35:296-300.
- 27 Chen G, Magis AT, Xu K, Park D, Yu DS, Owonikoko TK, Sica GL, Satola SW, Ramalingam SS, Carraway MS, Doetsch PW, Deng X: Targeting Mcl-1 enhances DNA replication stress sensitivity to cisplatin therapy. *J Invest* 2017;10.1172/jci92742.
- 28 Krajewski S, Bodrug S, Krajewska M, Shabaik A, Gascoyne R, Berean K, Reed JC: Immunohistochemical analysis of Mcl-1 protein in human tissues. Differential regulation of Mcl-1 and bcl-2 protein production suggests a unique role for Mcl-1 in control of programmed cell death *in vivo*. *Am J Pathol* 1995;146:1309-1319.
- 29 Lee WS, Park YL, Kim N, Oh HH, Son DJ, Kim MY, Oak CY, Chung CY, Lee HJ, Kim MY, Myung DS, Cho SB, Kim HS, Joo YE: Myeloid cell leukemia-1 is associated with tumor progression by inhibiting apoptosis and enhancing angiogenesis in colorectal cancer. *Am J Cancer Res* 2015;5:100-113.
- 30 Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busch C, Zeiher AM: Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999;399:601-605.
- 31 Dimmeler S, Haendeler J, Nehls M, Zeiher AM: Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and caspase-3-like proteases. *J Exp Med* 1997;185:601-607.
- 32 Dimmeler S, Dernbach E, Zeiher AM: Phosphorylation of the endothelial nitric oxide synthase at ser-1177 is required for VEGF-induced endothelial cell migration. *FEBS Lett* 2000;477:258-262.
- 33 Jiang BH, Zheng JZ, Aoki M, Vande Woude F: Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. *Proc Natl Acad Sci U S A* 2000;97:1749-1753.
- 34 Singh K, Balligand JL, Fishbein TA, Smith MA, Kelly RA: Regulation of cytokine-inducible nitric oxide synthase in cardiac myocytes and microvascular endothelial cells. Role of extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2) and p38. *J Biol Chem* 1996;271:1111-1117.