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CPT1A regulates breast cancer-associated lymphangiogenesis via VEGF signaling

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ABSTRACT

Background: Lymphangiogenesis is critical for metastasis of a variety of cancers, including breast cancer. CPT1A (carnitine palmitoyltransferase 1a) has been reported to play a critical role in breast cancer progress. However, the molecular mechanism remains elusive.

Methods: In order to investigate the role of CPT1A in HDLEC cells, short hairpin RNA approach was utilized to knock down the CPT1A gene expression. We employed transwell and lymphatic vessel formation assay to examine invasion and lymphangiogenesis of HDLEC (Human dermal lymphatic endothelial cells). RT-qPCR and westernblot analyses were used to determine genes expression in HDLEC and breast cancer cells. Finally, we determined the relative rate of acetyl-CoA/CoA in shNC and shCPT1A HDLEC cells by LC–MS approach.

Results: Knockdown of CPT1A in breast cancer cells (MCF-7 and MDA-MB-231) abolished invasion and lymphangiogenesis of HDLEC cells. Mechanistically, CPT1A depletion suppressed the expression of VEGF-C and VEGF-D in MCF-7 and MDA-MB-231 cells. Interestingly, CPT1A knockdown in HDLEC cells exhibited attenuated expression of lymphangiogenic markers (podoplanin, VEGFR-3, VEGF-C, VEGF-D and PROX-1). Consistently, CPT1A -null HDLEC cells displayed compromised invasion and lymphangiogenesis compared with negative control. Further investigation revealed that CPT1A regulated VEGFR3 via acetyl-CoA mediated H3K9ac, which could be abrogated by supplement of acetate.

Conclusions: In present study, we revealed the mechanism by which CPT1A regulates breast cancer-associated invasion and lymphangiogenesis. Our findings provide insights into CPT1A -promoted breast tumor metastasis and provide rationale for understanding breast cancer metastasis.

1. Introduction

Lymphangiogenesis is defined as formation of new lymphatic vessels, which plays critical roles in normal tissue development, inflammation, wound healing and even tumor metastasis [1–3]. Lymphangiogenesis has been shown to be implicated into breast cancer metastasis and poor prognosis [4–6]. However, little is known about the molecular mechanism by which breast cancer reciprocally affects lymphangiogenesis.

CPT1A locates at outer mitochondrial membrane and involves transporting long-chain fatty acids into mitochondria for beta-oxidation, together with CPT2 (Carnitine palmitoyl transferase) [7,8]. In addition, CPT1A was reported to regulate injury-induced lymphangiogenesis via histone acetylation [9]. Therefore, we hypothesized that CPT1A was likely to influence breast cancer-associated lymphangiogenesis.

VEGF-C and VEGF-D are two members of VEGF family associated with lymph node metastasis or lymphatic vessel density in breast cancer [10,11]. More importantly, VEGF-C and VEGF-D are important and unique lymphangiogenic factors via interaction with their cognate receptor VEGFR-3, which is expressed predominantly in lymphatic endothelial cells [12–14]. To date, VEGF-C and VEGF-D have been confirmed as two key regulators for breast tumor-associated lymphangiogenesis and tumor metastasis [15].

Here, we revealed that CPT1A1A contributes to breast cancer-induced invasion and lymphangiogenesis of HDLEC cells through VEGF-C/VEGF-D/VEGFR-3 signaling. Our findings provide insights into how CPT1A influences breast cancer-related lymphangiogenesis, thereby helping develop drugs for treating breast cancer via targeting CPT1A.

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2. Materials and methods

2.1. Cell lines

MCF-7 (human nonmetastatic breast cancer cell line), MDA-MB-231 (human highly metastatic breast cancer cell line) and 293 T (human immortalized embryonic kidney cell, well-recognized cell line for virus package due to high transfection efficiency) were purchased from ATCC and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (100 U/ml)/streptomycin (100 U/ml) at 37 °C, 5% CO_2. Human Dermal Lymphatic Endothelial Cells (HDLEC) was obtained from PromoCell (HeidelbergGermany) and Endothelial Cell Growth Medium (ECGM) (PromoCell). The conditioned medium (CM) from indicated treated cancer cells grown in DMEM supplemented with 10% FBS for 72 h was collected. HDLEC was seeded in six-well plates, and then treated with a 1:2 mixture of CM and fresh 10% FBS ECGM medium for indicated time.

2.2. Plasmid transfection and lentivirus package

The short hairpin RNAs (shRNAs) were cloned into PLKO.1 vector. To generate lentiviruses, the packaging vectors (pPAX2 and pVSVG) and transfer vector were co-transfected into 293 T cells. The supernatant was harvested at 24 h and 48 h after transfection. For virus infection, the virus supernatant was added to medium at 1:5 ratio, after 24 h, 2 u g/ml puromycin was employed to select the positive cell.

2.3. Transwell invasion assay

The cells were transfected with CPT1A shRNA or scramble. After 24 h, cells were starved in medium without serum for another 12 h and then digested with trypsin. Collected cells were seeded on the top chamber of 24 well transwell culture inserts (promega). Invasion assays were performed using the BD Bio-Coat Matrigel invasion assay system (BD Biosciences) according to the manufacturer's instructions. The nontile or noninvasive cells were removed, while the lower side of the filter were stained with 0.005% crystal violet and counted.

2.4. HDLEC tube formation assay

This assay was performed with HDLEC cells under different treatment conditions on GFR Matrigel. Matrigel was thawed and diluted with cold sterile PBS at 1:1 ratio, and used to coat 24-well culture plates (0.25 ml/well) and left at 37 °C for 6 h. After polymerization, about 50,000 cells/well suspended in DMEM medium were added to each well. Pictures of 10–15 random fields were captured in various experiments using a Leica Microscope EC3 camera and representative pictures were shown. The quantitative analysis of total tube lengths were performed using ImageJ software.

2.5. Western blot

Cells were harvested and rinsed with 1 × PBS buffer, then lysed by 2 × SDS loading buffer. The lysates were boiled at 100 °C for 5 min. About 40 u g of total proteins were loaded onto SDS-PAGE gel and resolved. After that, the proteins were transferred to NC membrane at 300 mA for 3 h. The protein-containing membrane was blocked with 5% non-fat milk in 1 × TBST for 1 h at room temperature, the membrane was then incubated with primary antibodies at 4 °C overnight. The next day, the membrane was washed with 1 × TBST for 3 times, 5 min each time. The membrane was incubated with secondary antibodies at RT for 1 h. Finally, the membrane was incubated with ECL solution and then exposed. The following antibodies were used: anti-CPT1A (cell signaling technology, #12252, 1:1000), anti-MMP2 (cell signaling technology, #40994, 1:1000), anti-MMP9 (cell signaling technology, #13667, 1:1000), anti-Vimentin (cell signaling technology, #5741, 1:1000), anti-E-cadherin (cell signaling technology, #3195, 1:1000), anti-VEGF-A (Abcam, ab46154, 1:500), anti-VEGF-B (cell signaling technology, #2463, 1:1000), anti-VEGF-C (cell signaling technology, #2445, 1:1000), anti-VEGF-D (Novus Biologicals, NB110-60973, 1:1000), anti-VEGFR-3 (cell signaling technology, #3408, 1:1000), anti-podoplanin (cell signaling technology, #9047, 1:1000), anti-β-actin (Proteintech, 60008-1-Ig, 1:2000).

2.6. RT-qPCR

We extracted the total RNA by Trizol method. Cells were lysed with Trizol buffer and then add chloroform to the mixture. The resultant was centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to new EP tube and mixed with equivalent volume of isopropanol, next, the resultant was centrifuged at 12,000 rpm for 10 min. Removing the supernatant and add 75% ethanol to wash the pellet and centrifuge. Finally, discard the ethanol and dry the pellet, use 20–30 ul Rnase-free H_2O to resolve the RNA.

For reverse transcription, 1 μg of total RNA was used for reverse transcription according to manufacturer instruction (TAKARA PrimeScript Kit). For real time PCR, we used SYBR as probe dye and detected the signal. GAPDH was used as internal control. The following primers were used:

CPT1A -QPCR-F: CTGGACAATACCTCGGAGCC
CPT1A -QPCR-R: AACGTCACAAAGAACGCTGC
VEGF-C-QPCR-F: AGACTCAATGCATGCCACG
VEGF-C-QPCR-R: TTGAGTCATCTCCAGCATCC
VEGF-D-QPCR-F: GCTGTTGCAATGAAGAGAGC
VEGF-D-QPCR-R: TCTTCTGTTCCAGCAAGTGG
VEGFR-3-QPCR-F: ATTCACATCGGCAACCACCT
VEGFR-3-QPCR-R: CGCAGGAAGTTGGAGAGGTT
PROX-1-QPCR-F: GAGATGTGCGAGCTAGACCC
PROX-1-QPCR-R: CACAGTGTCCACAACTTGCG
podoplanin-QPCR-F: AGGTGCCGAAGATGATGTGG
podoplanin-QPCR-R: TTGGACTTTGTTCTTGCGCG
GAPDH-QPCR-F: GAGTCAACGGATTTGGTCGT
GAPDH-QPCR-R: TTGATTTTGGAGGGATCTCG

Mass spectrometry for detection of acetyl-CoA and CoA

Cells were seeded in 6-wellplates and collected in 300 μl 1% PCA per well (Perchloric acid, Sigma). Analytes were separated on a Dionex Ultimate 3000UPLC (Thermo Scientific) equipped with a reversed-phase Atlantis dC18 column(2.1 mm × 150 mm, pore size 5 μm, Waters) in-line connected to a Q-Exactive Orbi TRAP mass spectrometer (Thermo Scientific). The column was thermostatted at 37 °C, and samples were kept at 4 °C. The following procedures were performed as previously described [9].

2.7. Statistical analysis

Each experiment was performed for three times, all values were presented as mean ± SD, comparisons of parameters were performed using the two-tailed unpaired student's *t*-test. $^*P < 0.05$ was considered statistically significant.

3. Results

3.1. CPT1A knockdown in breast cancer attenuates invasion and lymphangiogenesis of HDLEC cells

To investigate the role of CPT1A in breast cancer-associated lymphangiogenesis, we generated CPT1A -depleted MCF-7 and MDA-MB-231 cells using shRNAs approach. Westernblot showed CPT1A level was markedly decreased in MCF-7 and MDA-MB-231 transfected with shRNA against CPT1A compared to that of shNC (Fig. 1A). Next, we performed transwell assay to investigate whether CPT1A played a role