# Supplementary Information

**Title:** Regulation of IL-6 Signaling by miR-125a and let-7e in Endothelial Cells Controls Vasculogenic Mimicry Formation of Breast Cancer Cells

**Authors' names:** Youngsook Park[1], Jongmin Kim[1, 2]*

**Affiliation:**

[1]Division of Biological Sciences, Sookmyung Women's University, Seoul, 04310, Korea

[2]Research Institute for Women′s Health, Sookmyung Women′s University, Seoul 04310, Republic of Korea

**Running Title:** Regulation of IL-6 signaling by microRNAs

**Keywords:** vasculogenic mimicry, endothelial cell, IL-6 signaling, microRNA, chemoresistance

**Corresponding Author's Information:**

*To whom correspondence should be addressed:

Jongmin Kim, Ph.D.

Division of Biological Sciences, Sookmyung Women's University, Seoul, 04310, Korea

TEL: +82-2-710-9553

FAX: +82-2-2077-7322

E-mail: jkim@sookmyung.ac.kr

## SUPPLEMENTATY MATERIALS AND METHODS

### Luciferase reporter assay

Human target genes containing predicted miR-125a or let-7e binding seed sequences were cloned into *NotI* and *XhoI* sites of the psiCHECK2 vector (Promega) downstream of the renilla luciferase coding region. The target gene miRNA binding seed sequences were designed as follows:

IL-6 let-7e binding seed sequences: TACCTC mutated to TAAAGC

IL-6R let-7e binding seed sequences: TACCTC mutated to TGCATC

IL-6R miR-125a binding seed sequences: CTCAGGG mutated to CTTGAGG

STAT3 let-7e binding seed sequences: CTACCTC mutated to CTGATTC

STAT3 miR-125a binding seed sequences: CTCAGGG mutated to CTAGTGG.

Mutations were induced using the QuikChange II Site-Directed Mutagenesis Kit (Agilent). HEK-293 cells ($2 \times 10^5$ cells/ml) were transfected with the luciferase reporter constructs, and either miR-125a, let-7e, or negative control miRNA using lipofectamine 2000 (Invitrogen). After 28 h of incubation, luciferase activity was measured using the Dual-Luciferase Reporter Assay kit (Promega) according to the manufacturer's instructions.

### Quantitative reverse transcription PCR

Total RNA was isolated using the miRNeasy RNA isolation kit (Qiagen) according to the manufacturer's instructions. RNA was reverse transcribed using the TaqMan MiRNA Reverse Transcription Kit (Life Technologies). Quantitative reverse transcription PCR for the detection of miRNA expression was performed using TaqMan Universal Master Mix II, no UNG (Life Technologies). U6 small nuclear RNA was used as an internal control. Purified

RNA was reverse transcribed using the qPCRBIO cDNA Synthesis Kit (PCR BIOSYSTEMS) to prepare mRNA. For the detection of mRNA, RNA was reverse transcribed using the qPCRBIO cDNA Synthesis Kit (PCRBIOSYSTEMS). Quantitative RT-PCR was performed using the qPCRBIO SyGreen Mix Hi-ROX (PCRBIOSYSTEMS). We used oligonucleotide primer sequences specific for *IL-6* (5'-AGA CAG CCA CTC ACC TCT TCA G-3' and 5'-TTC TGC CAG TGC CTC TTT GCT G-3'), *IL-6R* (5'-GAC TGT GCA CTT GCT GGT GGA T-3' and 5'-ACT TCC TCA CCA AGA GCA CAG C-3'), *STAT3* (5'- CTT TGA GAC CGA GGT GTA TCA CC-3' and 5'-GGT CAG CAT GTT GTA CCA CAG G-3'), and *18S* (5'-GCC TCA CTA AAC CAT CCA ATC GG-3' and 5'-ACC CGT TGA ACC CCA TTC GTG A-3'). Data were normalized to the internal control (Ribosomal 18S RNA). All experiments were done in triplicates.

**Western blotting**

Cells were harvested and then lysed using RIPA buffer (Gendepot) supplemented with a protease and phosphatase Inhibitor cocktail (Roche). After being lysed, extracts were centrifuged at 13,000 rpm, 4 °C for 15 min. Protein concentration of the lysates was measured using the Pierce BCA Protein Assay kit (Thermo Scientific). Equal amounts of protein samples were loaded on a polyacrylamide gel and separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinyl difluoride membranes (Millipore). Blots were incubated with the following primary antibodies: IL-6Rα (SC-661), STAT3 (SC-482), phospho-STAT3 (CS-9131), and GAPDH (CS-2118; as a loading control) overnight at 4 °C. Blots were incubated with HRP-conjugated secondary antibodies (1:3000, Cell Signaling) and developed using the enhanced chemiluminescence detection method (Thermo Scientific).

**Three-dimensional culture assay**

MDA-MB-231 cells were trypsinized and resuspended with IL-6 (100 ng/ml), treated serum free DMEM, or HUVECs conditioned culture medium (HUVECs incubated for 24 h with 10 μM cisplatin in EBM2 serum free media with concurrent overexpression of the negative control miRNA or miR125a/let-7e). Cells were seeded at a density of $0.8 \times 10^5$ cells/ml on Matrigel matrix (Growth Factor Reduced, phenol-free; BD Biosciences) coated 24-well plates. HUVEC cells were seeded at a density of $1 \times 10^5$ cells/ml on 6-well plates and transfected with negative control miRNA or miR125a/let-7e using RNAimax (Invitrogen). The next day, cells were trypsinized and seeded at a density of $1 \times 10^5$ cells/ml per well on Matrigel matrix coated 24-well plates. Cells were observed 12-24 h after plating as designated for each experiment. Average tube length was analyzed using the ImageJ software.

**Adhesion assay**

THP-1 cells were grown in RPMI-1640 (Roswell Park Memorial Institute-1640) medium containing 10 % FBS and 1% penicillin-streptomycin. HUVEC cells were seeded at a density of $1 \times 10^5$ cells/ml on 6-well plates and transfected with negative control miRNA or miR125a/let-7e using RNAimax (Invitrogen). The next day, HUVEC cells were trypsinized and seeded at a density of $2 \times 10^5$ cells/ml per well on 48-well plates. Subsequently, the HUVECs were pre-incubated with TNF-α (100 ng/ml) or IL-6 (100 ng/ml) for 16 h prior to co-culture. THP-1 cells were labeled with 10 μM BCECF/AM (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester ; Invitrogen) for 30 min. Labeled THP-1 cells were seeded at a density of $1 \times 10^5$ cells/ml into a confluent culture of HUVECs and incubated for 45 min. Next, the co-cultured cells were washed three times with warm PBS

(phosphate-buffered saline). Bound THP-1 cells were lysed with Tris-HCL 50 mM + 0.1 % SDS lysis buffer, and images were acquired at 485 nm and 538 nm using an emission fluorescence microscope.

**Statistical analysis**

All experiments were performed independently, at least three times and all data are presented as the mean ± standard error of the mean (SEM) from three independent assays. $P<0.05$ was considered statistically significant.