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

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Running Title: Regulation of IL-6 signaling by microRNAs

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

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ABSTRACT

The role of tumor-proximal factors in tumor plasticity during chemoresistance and metastasis following chemotherapy is well studied. However, the role of endothelial cell (EC) derived paracrine factors in tumor plasticity, their effect on chemotherapeutic outcome, and the mechanism by which these paracrine factors modulate the tumor microenvironment are not well understood. In this study, we report a novel mechanism by which endothelial miR-125a and let-7e-mediated regulation of interleukin-6 (IL-6) signaling can manipulate vasculogenic mimicry (VM) formation of MDA-MB-231 breast cancer cells. We found that endothelial IL-6 levels were significantly higher in response to cisplatin treatment, whereas levels of IL-6 upon cisplatin exposure remained unchanged in MDA-MB-231 breast cancer cells. We additionally found an inverse correlation between IL-6 and miR-125a/let-7e expression levels in cisplatin treated ECs. Interestingly, *IL-6,* IL-6 receptor (*IL-6R*), and signal transducer and activator of transcription 3 (*STAT3*) genes in the IL-6 pathway are closely regulated by miR-125a and let-7e, which directly target its 3′ untranslated region. Functional analyses revealed that endothelial miR-125a and let-7e inhibit IL-6-induced adhesion of monocytes to ECs. Furthermore, conditioned medium from cisplatin treated ECs induced a significantly higher formation of VM in MDA-MB-231 breast cancer cells as compared to that from intact ECs; this effect of cisplatin treatment was abrogated by concurrent overexpression of miR-125a and let-7e. Overall, this study reveals a novel EC-tumor cell crosstalk mediated by the endothelial miR-125a/let-7e-IL-6 signaling axis, which might improve chemosensitivity and provide potential therapeutic targets for the treatment of cancer.

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

INTRODUCTION

Although many chemotherapeutic drugs have received remarkable initial responses, tumor recurrence is common. Chemoresistance and metastasis are the most common causes of cancer related death (1-3). In light of the limitations of current chemotherapeutic drugs, identification of novel mechanisms to overcome tumor recurrence, chemoresistance, and metastasis is critical.

To date, most studies have focused on the role of tumor-proximal factors in tumor recurrence and chemoresistance following chemotherapy (1, 4-6). Despite the support provided by the surrounding tumor microenvironment to cancer cells, the role of tumor stromal cells such as endothelial cells (ECs) in chemotherapeutic outcomes is still poorly understood. Healthy vascular ECs play an essential role in the maintenance of vascular homeostasis in response to various stimuli (7-9). In contrast, endothelial dysfunction caused by chemotherapeutic drugs contributes to tumor growth and survival (10, 11). However, it is not well understood how EC-derived paracrine factors affect endothelial dysfunction following chemotherapy, and how they influence tumor plasticity which eventually causes chemoresistance. Vasculogenic mimicry (VM) refers to the formation of tumor cell-lined endothelium-like vessels, which is known to provide blood supply for tumor growth and represents a key link connecting the aggressiveness of cancer and metastasis with short survival of patients (12-15). In addition, emerging evidence shows that VM plays key roles in tumor plasticity during drug resistance following chemotherapy (16, 17).

Interleukin-6 (IL-6) signaling plays key roles in the development of chemoresistance and in recurrence of various cancers. (18-20). Thus, *IL-6,* IL-6 receptor (*IL-6R*), signal transducer and activator of transcription 3 (*STAT3*), and *gp130* genes in the IL-6 signaling pathway have

74 been considered as key therapeutic targets for the treatment of many cancers (18-21). IL-6 is
75 secreted by tumor cells or surrounding tumor microenvironments, such as ECs, macrophages
76 and fibroblasts, and, orchestrates tumor progression in an autocrine or paracrine manner (11,
77 21). While many studies have demonstrated that the role of tumor cell-derived IL-6 is
78 associated with chemoresistance in various tumor types, relatively few studies have
79 investigated the role of EC-derived IL-6 in tumor plasticity and its effect on
80 chemotherapeutic outcome. In addition, it is not clear whether any microRNA (miRNA)
81 mediated regulatory mechanisms are involved in IL-6 production and whether they have an
82 effect on chemotherapeutic outcome in ECs.
83
84 In this study, we sought to define the role of the EC-derived paracrine factor, IL-6 in tumor
85 plasticity in a chemotherapeutic therapy, and to understand the role of miRNAs in IL-6
86 signaling mediated paracrine effects on tumor cells. We demonstrated that IL-6 levels are
87 upregulated as a consequence of decrease in miR-125a and let-7e expression in cisplatin
88 treated ECs, although IL-6 levels were not affected in cisplatin treated MDA-MB-231 breast
89 cancer cells, which suggests the importance of endothelium-derived IL-6. We found that
90 conditioned medium (CM) from cisplatin treated ECs induces significantly greater formation
91 of VM in MDA-MB-231 breast cancer cells, and might eventually cause drug resistance and
92 extensive metastasis. These findings reveal a novel mechanism by which miRNA-mediated
93 regulation of endothelial IL-6 signaling can manipulate VM formation through the platinum-
94 containing chemotherapeutic agent, cisplatin in breast cancer cells.
95
96

RESULTS

Cisplatin treatment induces IL-6 expression and inhibits miR-125a/let-7e expression in ECs.

To analyze the effect of a chemotherapeutic drug on IL-6 expression in the tumor microenvironment, we treated tumor cells and ECs with cisplatin and assessed expression levels of IL-6. Treatment of human umbilical vein ECs (HUVECs) with cisplatin led to significantly increased IL-6 expression levels (Fig. 1A), whereas IL-6 expression was not affected in cisplatin treated MDA-MB-231 breast cancer cells (Data not shown), suggesting a potential role for endothelium-derived paracrine IL-6 secretion in the microenvironment of breast cancer. Several molecular mechanisms have been shown to affect IL-6 expression, including a critical role for miRNA-mediated regulation in different cellular contexts, such as in macrophages (22) and preosteoblasts (23). The potential role of miRNAs as regulators of IL-6 expression in ECs is still unknown. Thus, to identify miRNAs that might regulate the expression of IL-6 in response to cisplatin in ECs, we first analyzed the expression of miRNAs in response to cisplatin in HUVEC and EC-enriched miRNAs, using publicly available miRNA expression profiling data (22, 23). The target prediction algorithm (TargetScan) was used to identify potential miRNAs targeting IL-6 and related genes (Fig. 1B). From these results, we found two miRNAs (miR-125a and let-7e) which are highly expressed in ECs and are expressed as a cluster. We next examined miR-125a and let-7e expression in cisplatin treated ECs. As shown in Fig. 1C, miR-125a and let-7e levels decreased in response to cisplatin treatment in ECs. These findings suggest that miR-125a and let-7e may be involved in the mechanism underlying the induction of IL-6 expression in response to cisplatin.

***IL-6, IL-6R and STAT3* genes from the IL-6 pathway are directly targeted by miR-125a/let-7e, and these miRNAs affect the phosphorylation of STAT3.**

To further investigate the relationship between miR-125a/let-7e and IL-6, we determined the effects of miR-125a and let-7e overexpression on IL-6 expression in HUVECs. Overexpression of miR-125a or let-7e led to significant downregulation of IL-6 expression, whereas inhibition of endogenous miR-125a or let-7e by anti-miRs in HUVECs led to upregulation of IL-6 expression (Fig. 2A). Next, we determined whether miR-125a and let-7e regulate IL-6 expression by binding directly to its 3′UTR. Let-7e was predicted to target the *IL-6* 3′UTR, while miR-125a was not (Fig. 1B). We generated luciferase constructs containing the 3′UTR of *IL-6* and found that let-7e overexpression led to a marked decrease in reporter activity. This response was abrogated with mutagenized *IL-6* 3′UTR (Fig. 2B). Overall, these results show that let-7e can directly target *IL-6*. However, further investigation is required to elucidate the molecular mechanism by which miR-125a inhibits IL-6 expression.

We further examined additional predicted targets of let-7e and miR-125a which might play an essential role in the IL-6 signaling pathway in ECs. As shown in Fig. 2C and 2D, overexpression or knockdown of miR-125a or let-7e had effects on IL-6R and STAT3 expression similar to those on IL-6 expression, although let-7e had no effect on *STAT3* mRNA levels, and we demonstrated that miR-125a and let-7e significantly reduced reporter activity of the 3′UTR of *IL-6R* and *STAT3,* though not that of the mutant forms (Fig. 2E). Finally, we speculated whether perturbation of miR-125a and let-7e expression affects the phosphorylation of STAT3, a key downstream mediator of IL-6 signaling (24). miR-125a and let-7e overexpression led to a robust decrease in STAT3 phosphorylation with exogenous IL-6 stimulation (Fig. 2F).

Endothelial miR-125a/let-7e suppresses IL-6-induced adhesion of monocytes to ECs and induces paracrine inhibition of VM formation by cisplatin-treated EC conditioned media.

Because the IL-6 signaling pathway plays a critical role in vascular functions such as inflammation and monocyte adhesion (25, 26), we examined the effect of miR-125a/let-7e on IL-6 induced adhesion of monocytes to ECs. miR-125 and let-7e overexpression significantly inhibited the IL-6 induced THP-1 cell adhesion to HUVECs (Fig. 3A), suggesting that this effect may be secondary to the targeting of IL-6R and STAT3. However, miR-125 and let-7e overexpression had no effect on the TNF-α induced THP-1 cell adhesion to HUVECs. These results indicate that miR-125 and let-7e might act specifically on the adhesion of monocytes to ECs in response to IL-6.

Next, to understand the role of EC derived paracrine IL-6 on tumor plasticity, we treated MDA-MB-231 breast cancer cells with IL-6, and examined the formation of VM using the well established *in vitro* tube formation assay. As shown in Fig. 3B, IL-6 significantly induced the formation of VM in MDA-MB-231 breast cancer cells, whereas *IL-6* knockdown led to a significant decrease in VM formation. Given that cisplatin treatment induces IL-6 expression and inhibits miR-125a/let-7e expression in ECs, we evaluated the role of IL-6 regulation by miR-125a and let-7e on tumor plasticity in response to cisplatin, and we subjected MDA-MB-231 breast cancer cells to control EC conditioned medium (CM) or cisplatin containing EC CM. We found that treatment with cisplatin containing EC CM resulted in a robust increase in VM formation compared to treatment with CM from control cells. This effect of cisplatin treatment was abrogated by concurrent overexpression of miR-125a and let-7e (Fig. 3C). Altogether, IL-6 signaling regulation by miR-125a and let-7e in ECs might modulate endothelial dysfunction and VM formation upon cisplatin treatment.

DISCUSSION

Previous studies have associated elevated IL-6 levels with poor overall survival and tumor progression in various cancers (27-29). In addition, IL-6 signaling plays a key role in the development of chemoresistance in various cancers (18-20). Vasculature is essential for cancer progression and reprograms the tumor microenvironment. In particular, ECs play a critical role in the maintenance of vascular homeostasis, whereas dysfunctional ECs contribute to the pathogenesis of various diseases and to tumor progression by secreting cytokines. (10, 11). Here, we describe miRNA-driven regulation of IL-6 signaling in ECs and show its involvement in the regulation of cell adhesion and in the formation of VM in an autocrine and paracrine manner in tumor microenvironments in response to cisplatin treatment.

Many studies have shown that miRNAs play key roles in the context of cancer and vascular biology (9, 10, 30-33). However, endothelial miRNA-mediated autocrine and paracrine regulation of chemotherapeutic outcome in tumor microenvironments is poorly understood. Previous reports show that miR-125a and let-7e are significantly downregulated in many cancers, and downregulation of these miRNAs leads to enhanced tumor progression, chemoresistance, and metastasis despite strong context dependence of the role of miR-125a and let-7e in cancer (34-40). Although miR-125a and let-7e are highly expressed in ECs (8, 41), and have been studied in different cellular contexts such as in immune cells, preosteoblasts, and in a variety of cancers (22, 23, 34, 39, 42, 43), their role in EC biology remains unclear. Therefore, it is important to understand the role or regulation of miR-125a and let-7e in tumors as well as in tumor microenvironments such as ECs. Our current findings provide three key aspects of highly enriched endothelial miRNAs, miR-125a and let-7e, which might act as important mediators for modulating tumor plasticity and thus affect the

chemotherapeutic outcome. First, we found that IL-6 and miR-125a/let-7e are inversely regulated by the platinum-containing chemotherapeutic agent, cisplatin in HUVECs. Second, we showed that miR-125a and let-7e directly target *IL-6, IL-6R* and *STAT3* genes from the IL-6 pathway and inhibit IL-6-induced adhesion of monocytes to ECs. Lastly, we found that miR-125a and let-7e suppress VM formation in MDA-MB-231 breast cancer cells using CM from cisplatin treated EC cultures by targeting IL-6 signaling. Together, our findings support the hypothesis that endothelium mediated paracrine cancer regulation affects chemotherapeutic outcome, and provide a novel molecular mechanism by which miRNA mediated EC paracrine factors modulate VM formation in breast cancer cells .

VM reflects tumor plasticity of aggressive tumor cells acquiring tumor cell-lined endothelium-like vessels, and is an independent blood supply system from endothelial vessels. Emerging evidence shows that the roles of VM in several cancers, are associated with tumor aggressiveness and poor patient survival, as well as tumor plasticity during chemoresistance and metastasis (12-16, 44). In light of the association of VM and tumor progression, coupled with ineffective anti-angiogenic drugs associated with the inhibition of VM (45, 46), identifying novel therapeutic targets that inhibit VM, might be effective to overcome chemoresistance and to eliminate tumor progression. However, the molecular mechanism underlying the formation of VM in tumor microenvironments remains unclear. Our current data provide a novel mechanism by which restoration of miR-125a and let-7e expression in cisplatin treated ECs can inhibit VM formation in MDA-MB-231 breast cancer cells, in a paracrine manner via targeting IL-6 signaling in ECs. These findings support the emerging role of dysfunctional EC-tumor cell crosstalk which likely plays a critical role in tumor progression and chemoresistance. Given these findings, future studies will be necessary to identify other paracrine factors in ECs affecting chemotherapeutic outcome. Furthermore, our ongoing studies to elucidate the precise role of miR-125/let-7e-IL-6 signaling axis in ECs,

221 both in chemotherapeutic drug induced endothelial dysfunction, and tumor progression in a
222 paracrine manner, will provide greater insights into tumor-EC interaction.
223
224 In conclusion, we show that downregulation of miR-125a and let-7e and the subsequent
225 activation of IL-6 signaling in ECs on chemotherapeutic drugs is closely associated with
226 cisplatin-induced VM formation and monocyte adhesion in tumor microenvironments. These
227 findings might promote the development of novel therapeutic candidate drugs, such that
228 chemotherapy combined with IL-6 inhibition might help to overcome chemoresistance
229 and ,thus, help in treatment of cancer.
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MATERIALS AND METHODS

An extended Materials and Methods section is available in the Supplementary Material.

Cell culture and Transfection

HUVECs (Human umbilical vein ECs; Lonza and Yale VBT Core) were maintained in EBM-2 basal medium supplemented with EGM-2 (Lonza) with 1 % penicillin/streptomycin (Welgene). HUVECs were grown to 70 % to 90 % confluency and used between passages 4 to 7 for all experiments. MDA-MB-231 (human breast adenocarcinoma cells) were maintained in DMEM (Dulbecco's modified Eagles medium; Welgene) supplemented with 10 % FBS (fetal bovine serum, Hyclone) and 1% penicillin-streptomycin (Welgene). HEK-293 cells (human embryo kidney cells) were maintained in DMEM supplemented with 10 % FBS and 1 % penicillin/streptomycin. Cells were maintained in a 5 % CO_2 incubator at 37 °C. siRNA (Bioneer), miRNA mimics and anti-miRs (miRVana; Ambion) were transfected into cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

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CONFLICTS OF INTEREST

The authors have no conflicting financial interests.

FIGURE LEGENDS

Figure 1. Cisplatin treatment upregulates IL-6 and reduces miR-125a/let-7e expression in HUVECs. (A) *IL-6* mRNA expression in response to cisplatin treatment (10 μM) in HUVECs. (B) Predicted target sequences of IL-6, IL-6R, and STAT3 3′ UTRs targeted by miR-125a and let-7e, and mutated sequences for disrupting miR-125a and let-7e recognition sequences. (C) Quantitative PCR showing expression of miR-125a and let-7e in response to cisplatin treatment (10 μM) in HUVECs. $*P < 0.05$, $***P < 0.001$ compared to controls. Error bars, s.e.m.

Figure 2. miR-125a and let-7e inhibit IL-6 signaling by directly targeting the *IL-6, IL-6R and STAT3* genes in HUVECs. (A) *IL-6* mRNA expression in response to overexpression of miR-125a or let-7e mimics, or inhibition of miR-125a or let-7e with anti-miRs in HUVECs. (B) Effect of let-7e overexpression on the luciferase reporter containing either the wild-type or mutagenized 3′ UTR of the human *IL-6* gene in HEK-293 cells. Luciferase activity for constructs with the wild-type (WT) and mutant (MT) 3′ UTR sequences. (C, D) IL-6R and STAT3 expression in response to overexpression of miR-125a or let-7e mimics, or inhibition of miR-125a or let-7e with anti-miRs in HUVECs. (E) Effect of miR-125a or let-7e overexpression on the luciferase reporter containing either the wild-type or mutagenized 3′UTR of the human *IL-6R* or *STAT3* gene in HEK-293 cells. Luciferase activity for constructs with the WT and MT 3′ UTR constructs. (F) STAT3 phosphorylation in response to IL-6 (100 ng/ml) in HUVECs with concurrent overexpression of miR-125a or let-7e. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to controls. Error bars, s.e.m.

Figure 3. Endothelial miR-125a/let-7e axis suppresses IL-6-induced adhesion of monocytes to ECs and regulates the development of vasculogenic mimicry in MDA-MB-

231 breast cancer cells in a paracrine manner. (A) IL-6 recombinant protein (100 ng/ml) increases adhesion of monocytes to HUVECs and miR-125a/let-7e overexpression suppresses IL-6-induced adhesion of monocytes to HUVECs. (B) Development of vasculogenic mimicry in response to IL-6 recombinant protein (100 ng/ml) or *IL-6* knockdown in MDA-MB-231 breast cancer cells. (C) Development of vascular mimicry of MDA-MB-231 breast cancer cells induced by cisplatin treated EC conditioned medium (CM) with concurrent overexpression of miR-125a and let-7e. $*P < 0.05$, $***P < 0.001$ compared to controls. Scale bar, 200 μm. Error bars, s.e.m.

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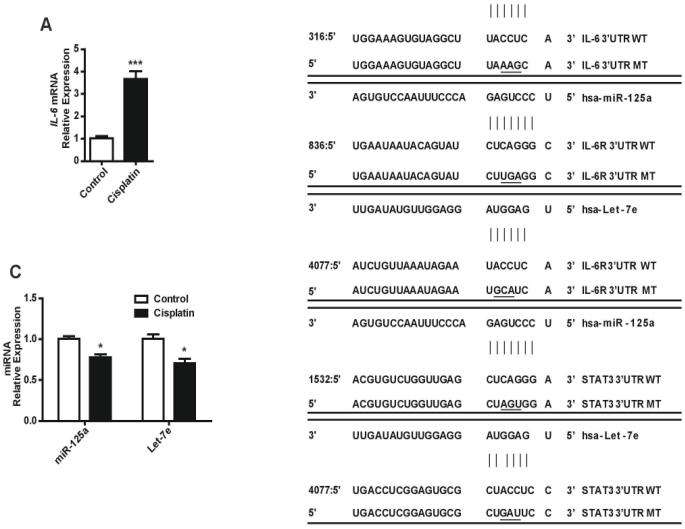

Fig. 1.

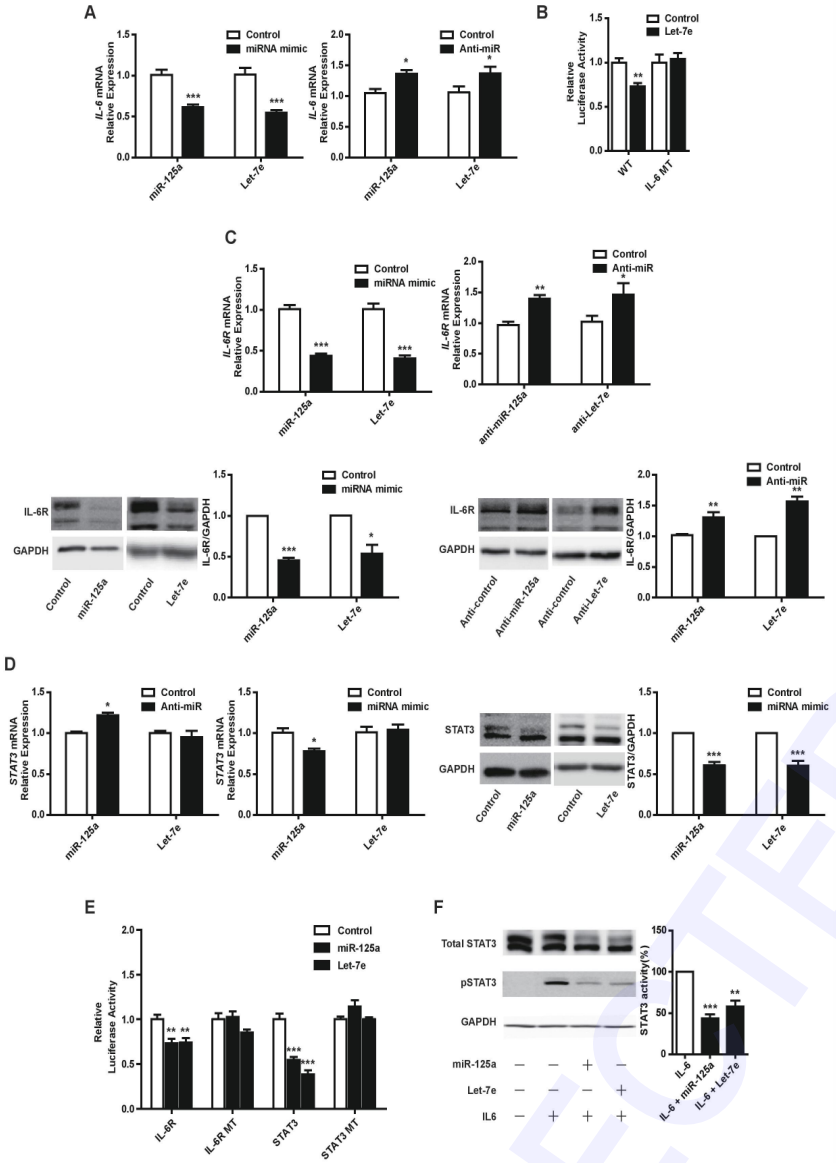

Fig. 2.

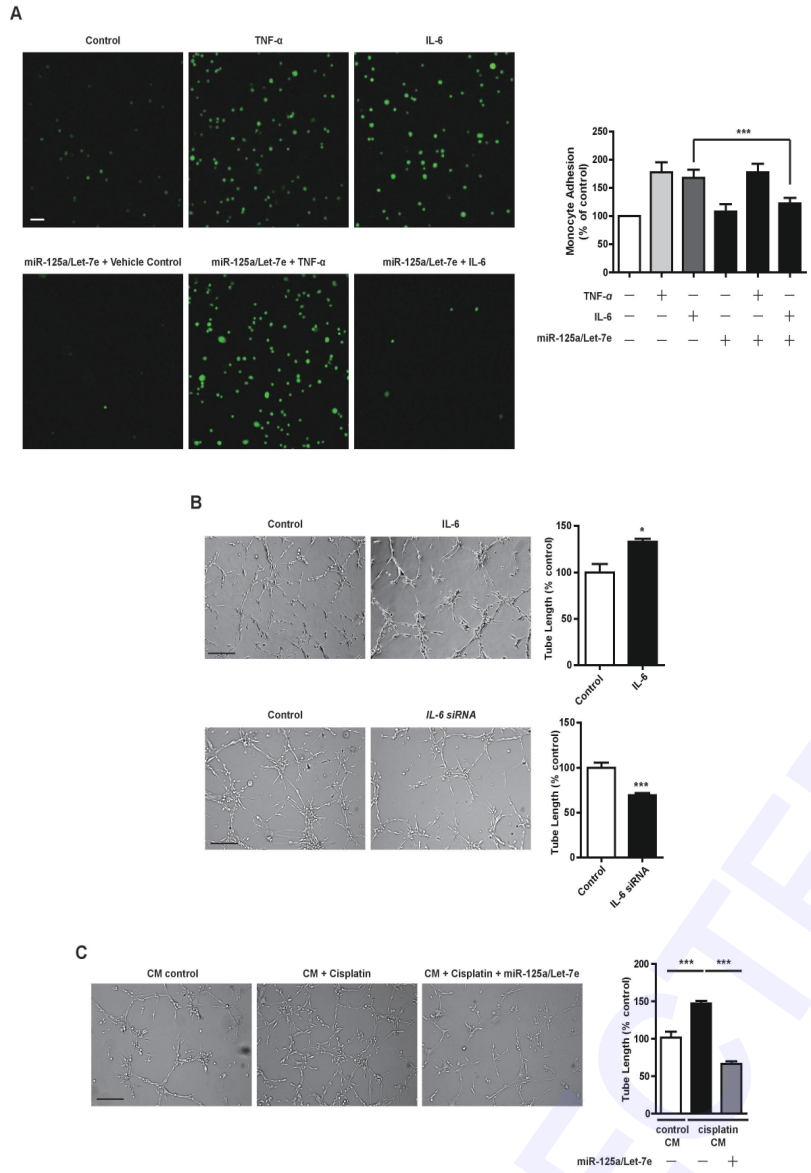

Fig. 3.

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

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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 (2×10^5 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 0.8×10^5 cells/ml on Matrigel matrix (Growth Factor Reduced, phenol-free; BD Biosciences) coated 24-well plates. HUVEC cells were seeded at a density of 1×10^5 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 1×10^5 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 1×10^5 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 2×10^5 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 1×10^5 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.