SUPPLEMENTAL MATERIALS

IL-6 contributes to deep vein thrombosis and is negatively regulated by miR-338-5p

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Major Resources Tables

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex
C57BL/6J Mice	Ribobio	C57BL/6J	male
C57BL/6J Mice	R&D system	C57BL/6J	male

Animal breeding

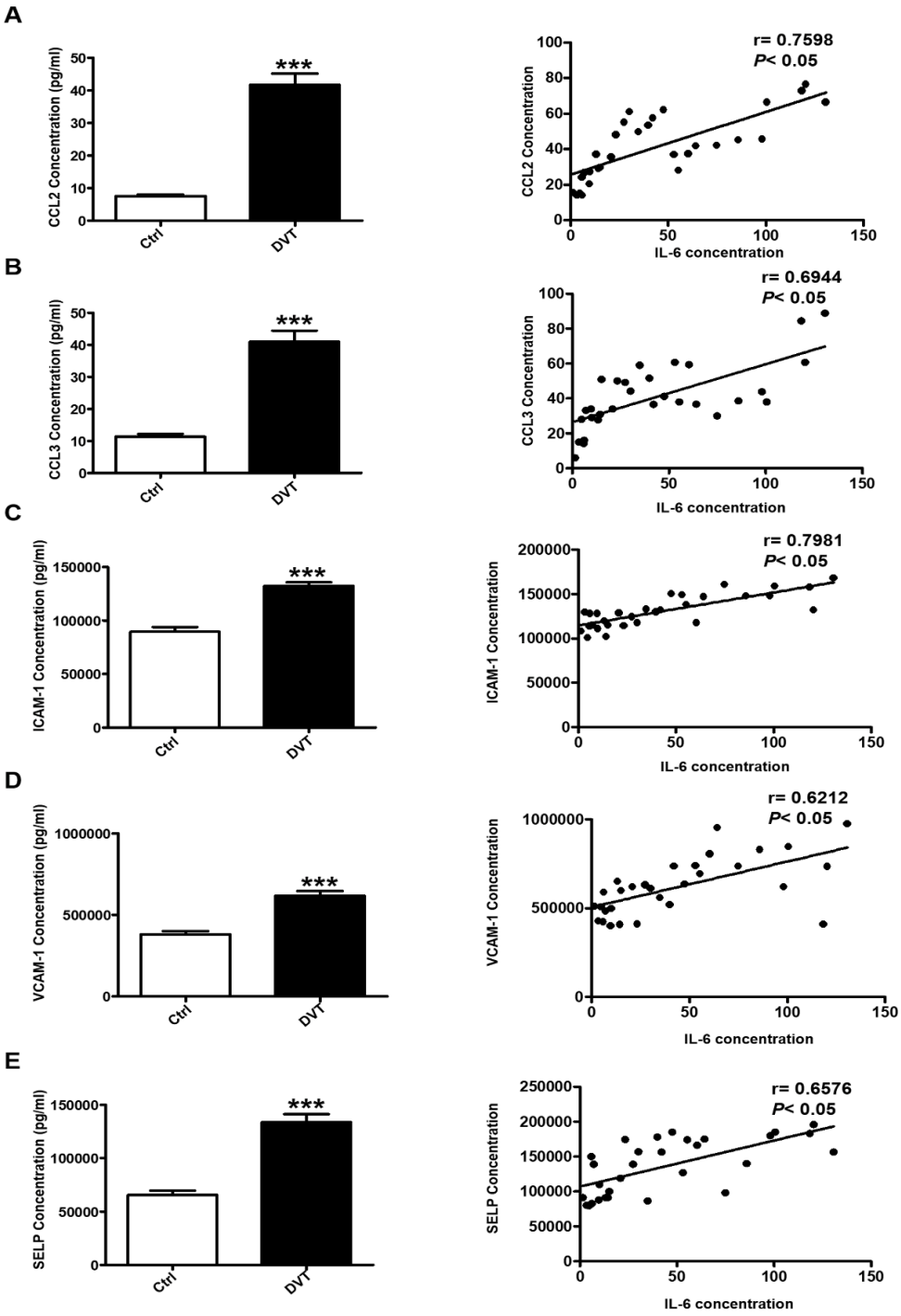
	Species	Vendor or Source	Background Strain	Other Information
Parent - Male	C57BL/6J Mice	SPF Biotechnology Company	C57BL/6J	None
Parent - Female	C57BL/6J Mice	SPF Biotechnology Company	C57BL/6J	None

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)
Rabbit Polyclonal IL-6 antibody	Proteintech	21865-1-AP	3.5 ug/ml	00071431
Mouse Anti-Rabbit IgG（H+L） Secondary Antibody, FITC Conjugate	BOSTER	BM2012	20 ug/ml	BST12F05A12
Mouse IL-6 Antibody	R&D system	MAB406	5ug/g	AHV2319021
Rat IgG1 Isotype Control	R&D system	MAB005	5ug/g	CAN1019031

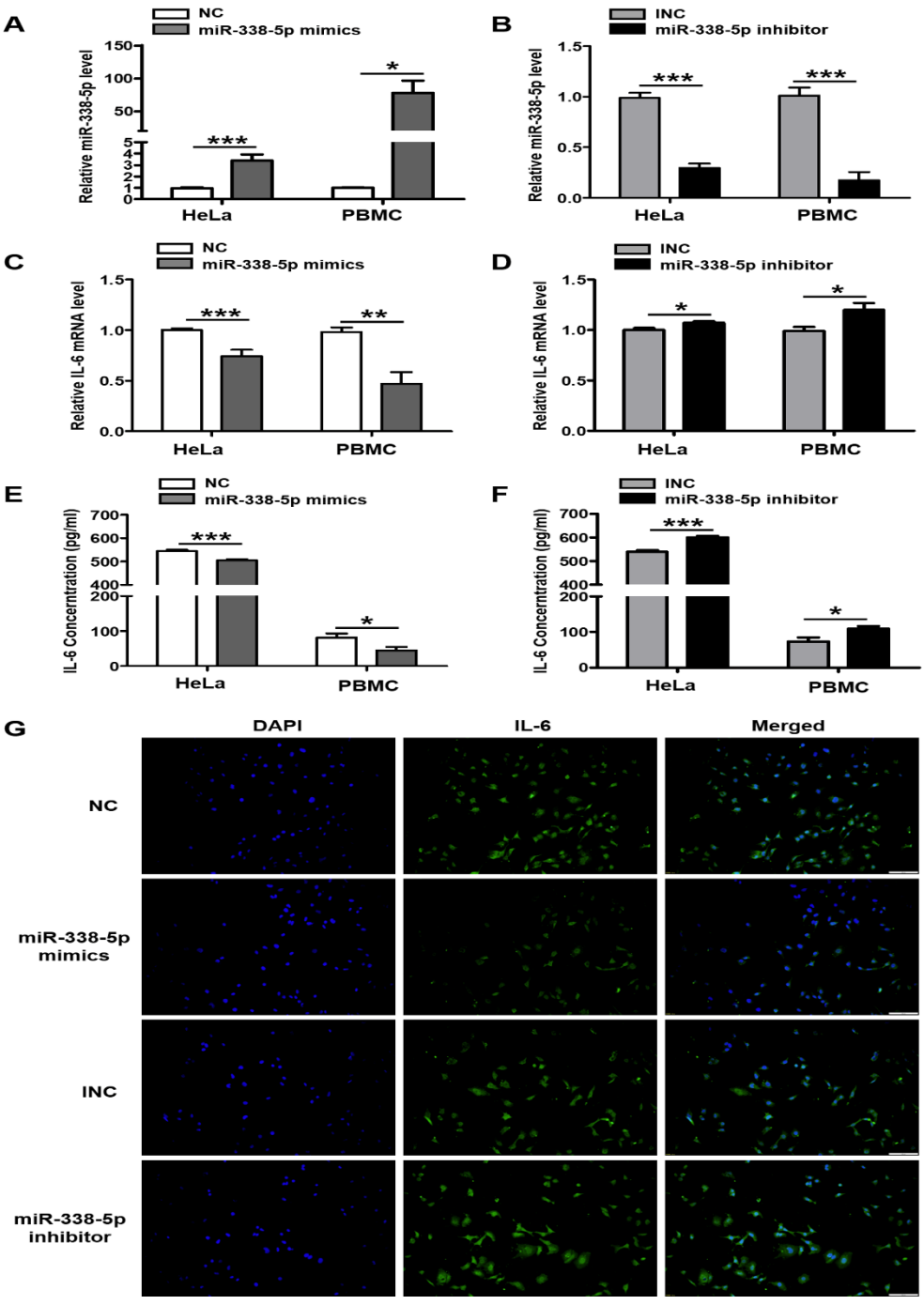
Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)
293T cells	Procell Life Science & Technology Co., Ltd	unknown
HeLa cells	Procell Life Science & Technology Co., Ltd	unknown
Human umbilical vein endothelial cells (HUVECs)	Procell Life Science & Technology Co., Ltd	unknown
Peripheral blood mononuclear cells (PBMCs)	Affiliated Hospital of Shandong University of Traditional Chinese Medicine	unknown



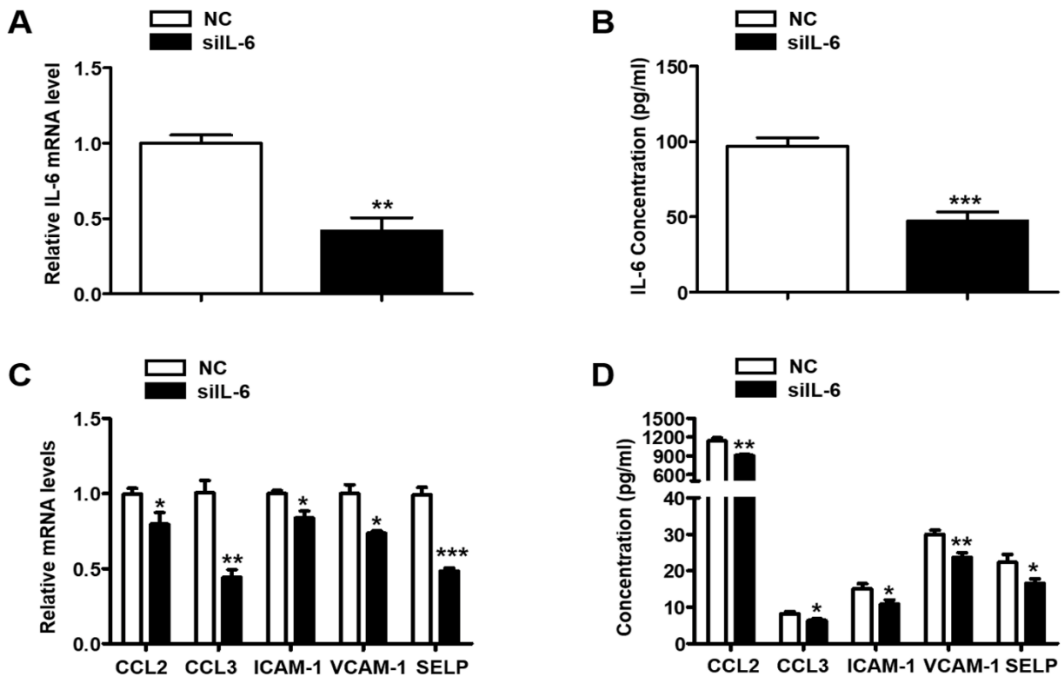
Supplemental Figure I. IL-6 is positively correlated to markers of vascular endothelial function in DVT.

(A-E) The expression levels of CCL2, CCL3, ICAM-1, VCAM-1, and SELP protein in the plasma from 30 DVT patients and 30 controls were determined by ELISA. The correlation between CCL2, CCL3, ICAM-1, VCAM-1, SELP, and IL-6 was analyzed using Pearson correlation analysis (n= 30). ****P*< 0.001.



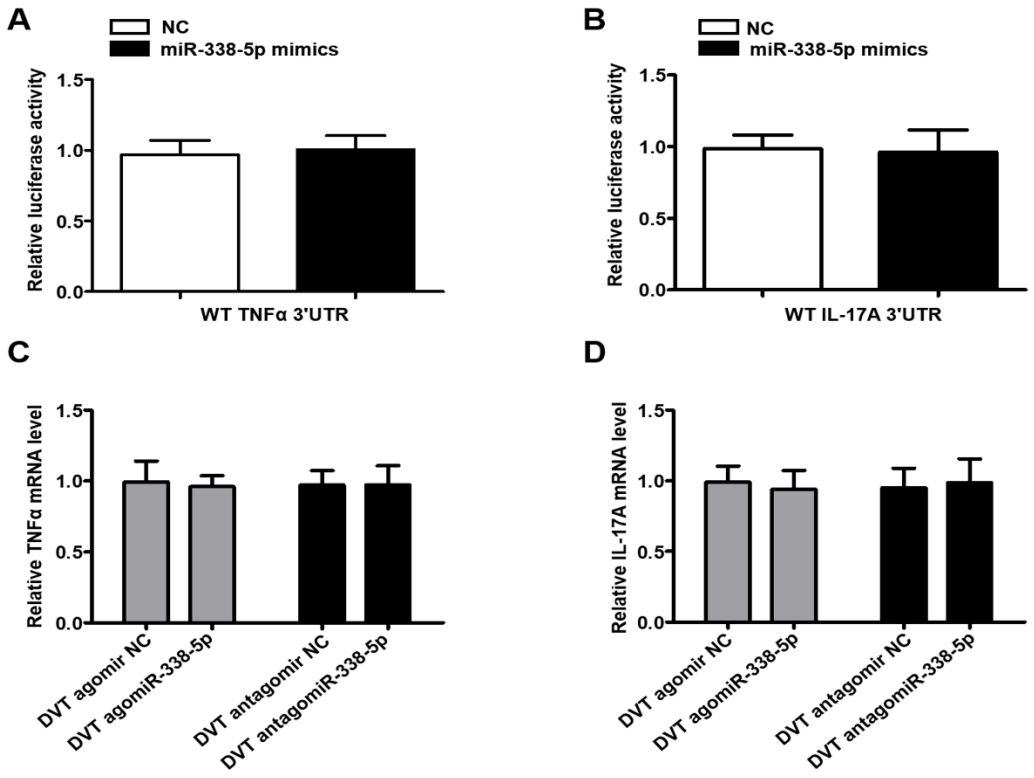
Supplemental Figure II. miR-338-5p negatively regulates IL-6 expression.

(A-B) The expression of miR-338-5p was detected by qRT-PCR in HeLa cells and PBMCs. (C-D) The expression level of *IL-6* mRNA after negative control (NC), miR-338-5p mimics, inhibitor negative control (INC), and miR-338-5p inhibitor transfection as detected by qRT-PCR in HeLa cells and PBMCs. (E-F) Expression of IL-6 protein was examined by ELISA in HeLa cells and PBMCs. (G) Expression of IL-6 in HUVECs were detected after transfection with NC, miR-338-5p mimics, INC, and miR-338-5p inhibitor by immunofluorescence staining (magnification, ×200). Scale bar= 100 μm. **P*< 0.05, ***P*< 0.01, ****P*< 0.001.



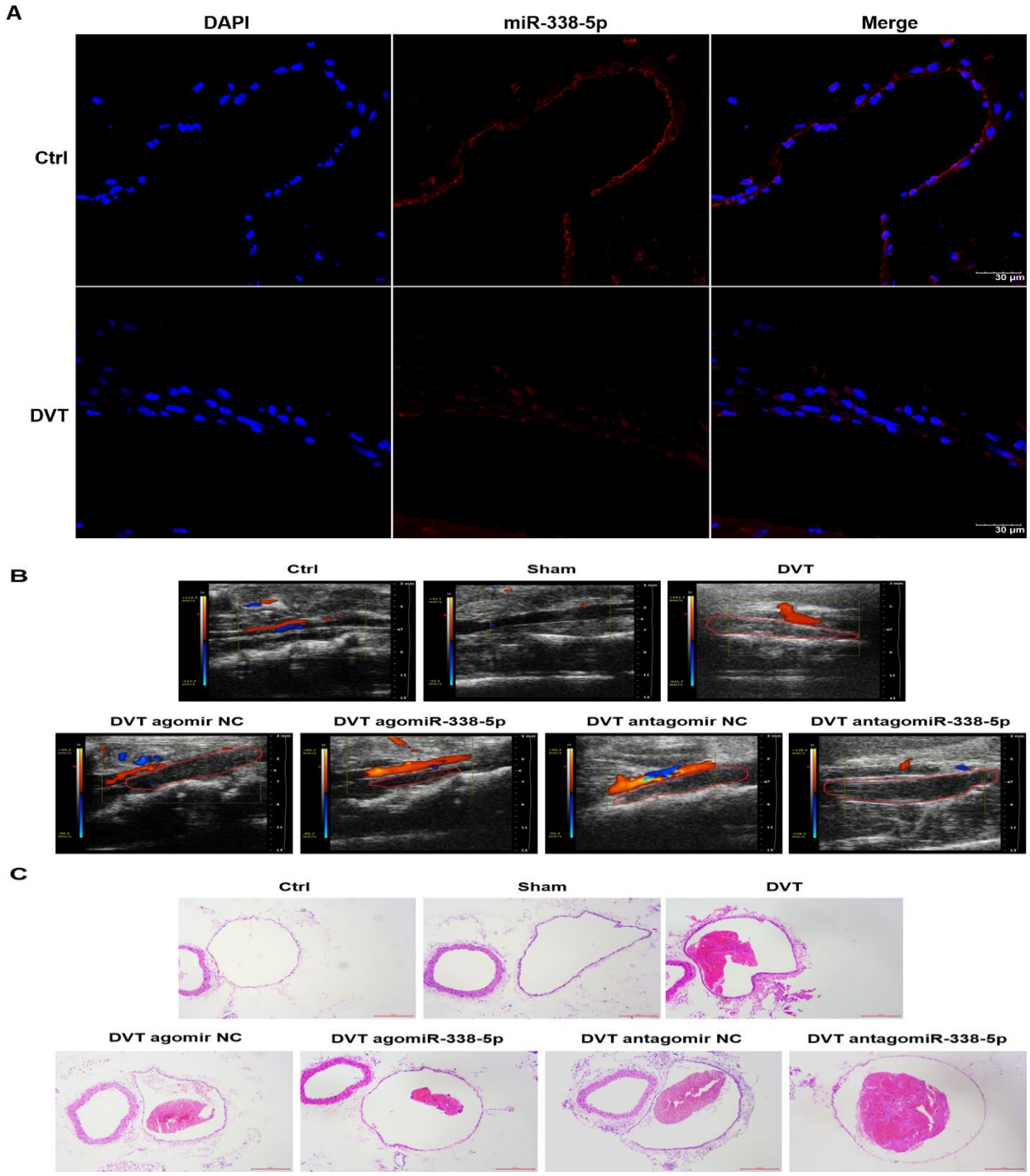
Supplemental Figure III. IL-6 knockdown decreases mRNA and protein levels of the markers for vascular endothelial function *in vitro*.

(A-B) The expression levels of IL-6 mRNA and protein were modulated by siRNA in HUVECs. (C-D) The levels of CCL2, CCL3, ICAM-1, VCAM-1 and SELP mRNA and protein were reduced when IL-6 was knocked down by siRNA in HUVECs. **P*< 0.05, ***P*< 0.01, ****P*< 0.001.



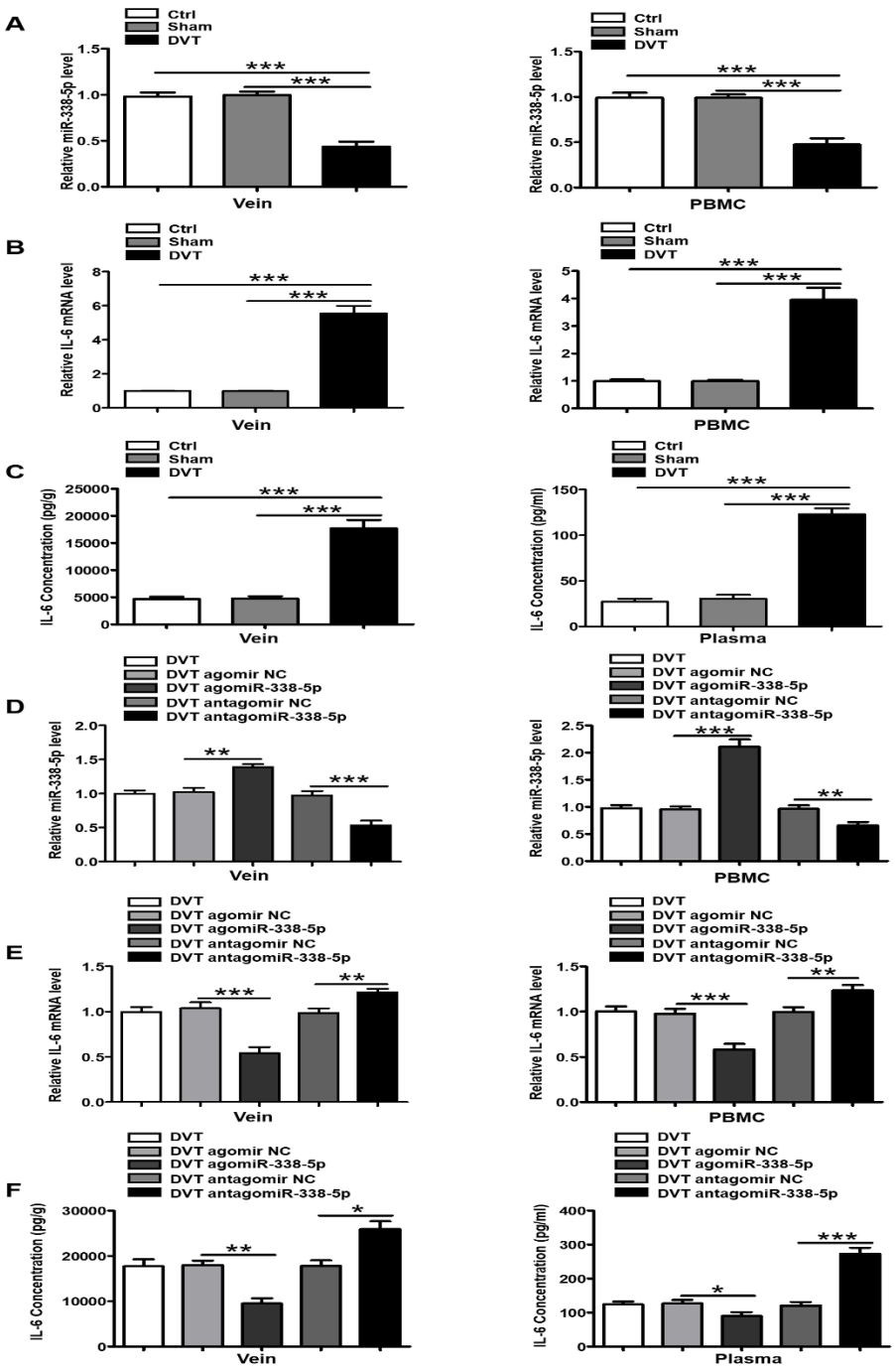
Supplemental Figure IV. miR-338-5p does not target *TNFα* or *IL-17A*.

(A-B) The luciferase activity was determined by co-transfecting the vectors (*TNFα* or *IL-17A* 3'-UTR-WT) combined with NC or miR-338-5p mimics into 293T cells. (C) *TNFα* mRNA expression was determined by qRT-PCR in PBMCs of DVT mice treated with agomiR-338-5p (n= 7) or antagomiR-338-5p (n= 12). (D) *IL-17A* mRNA expression was determined by qRT-PCR in PBMCs of DVT mice treated with agomiR-338-5p (n= 7) or antagomiR-338-5p (n= 12).



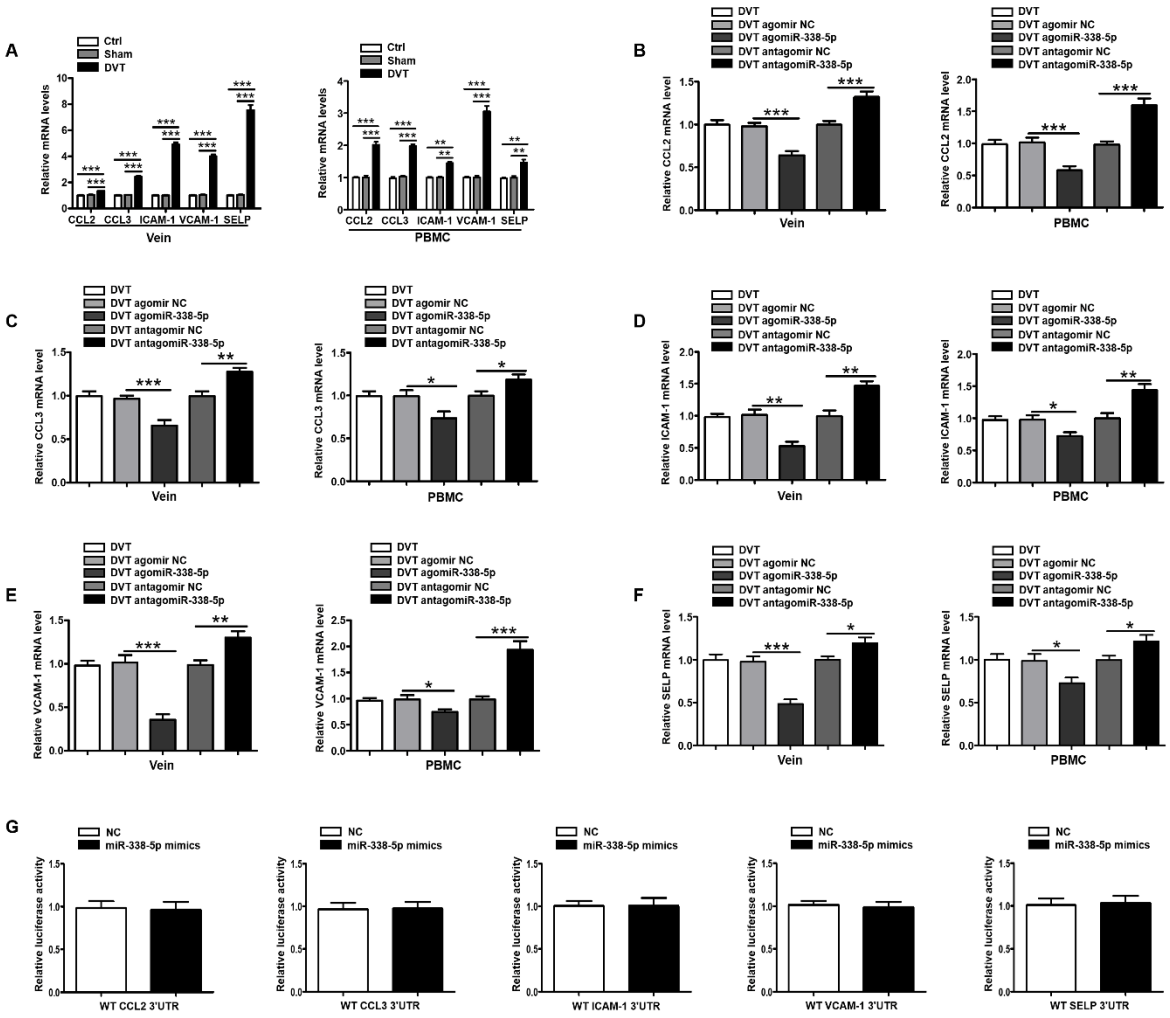
Supplemental Figure V. The role of miR-338-5p in DVT.

(A) Confocal microscopy for miR-338-5p expression in vascular tissues (miR-338-5p, red; DAPI, blue; magnification, ×600). Scale bars= 30 μm. (B) Representative images of thrombi in each treatment group detected by vascular ultrasound at 48 h post-operation. (C) H&E staining of serial cross sections of inferior vena cava (IVC) from control, sham, DVT mice, and DVT mice treated with agomir NC, agomiR-338-5p, antagomir NC, or antagomiR-338-5p at 48 hours (magnification, ×100). Scale bars= 200 μm.



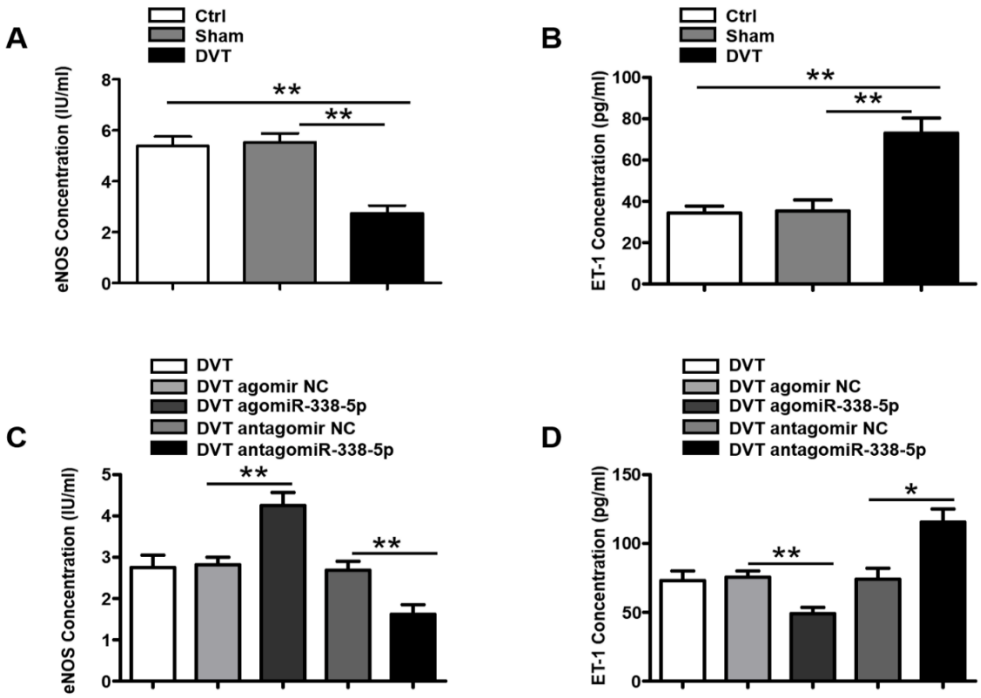
Supplemental Figure VI. The regulatory effect of miR-338-5p overexpression on IL-6 *in vivo*.

(A-B) The expression levels of miR-338-5p and *IL-6* mRNA were detected by qRT-PCR in vascular tissue and PBMCs of control, sham, and DVT animal group (n= 10-15/group). (C) IL-6 protein levels were determined by ELISA in vascular tissue and plasma of control, sham, and DVT animal group (n= 10-15/group). (D-E) The expression levels of miR-338-5p and *IL-6* mRNA were detected by qRT-PCR in vascular tissue and PBMCs of each treatment group (n= 7-12/group). (F) IL-6 protein levels were determined by ELISA in vascular tissue and plasma of each treatment group (n= 7-12/group). **P*< 0.05, ***P*< 0.01, ****P*< 0.001.



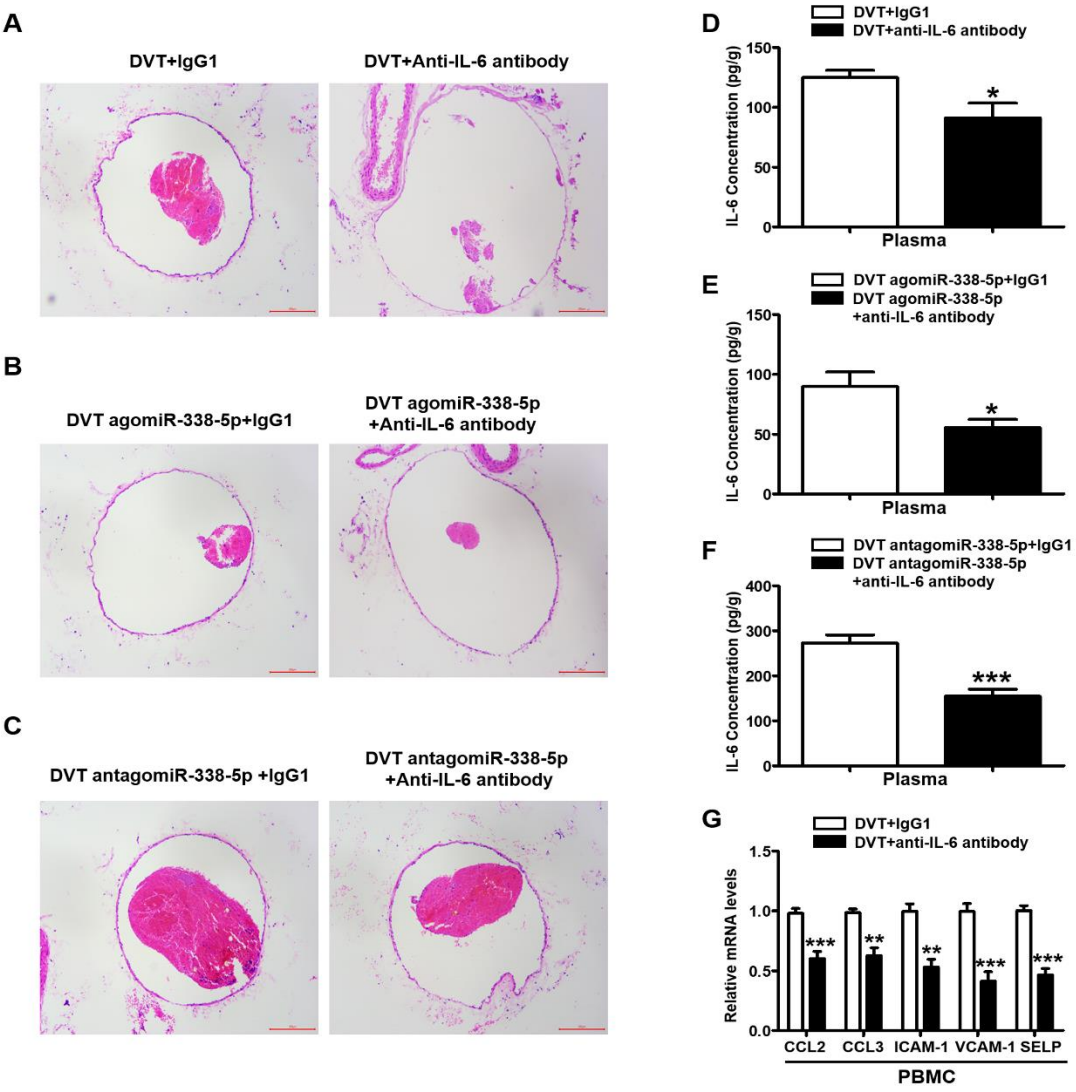
Supplemental Figure VII. The regulatory effect of miR-338-5p overexpression on vascular markers of endothelial function *in vivo*.

(A) Expression of *CCL2*, *CCL3*, *ICAM-1*, *VCAM-1*, and *SELP* was detected by qRT-PCR in vascular tissue and PBMCs of control, sham, DVT animal group (n= 10-15/group). (B-F) *CCL2*, *CCL3*, *ICAM-1*, *VCAM-1*, and *SELP* mRNA levels were determined by qRT-PCR in vascular tissue and PBMCs of DVT animal group and each treatment group (n= 7-12/group). (G) The luciferase activity was determined by co-transfecting the vectors (*CCL2*, *CCL3*, *ICAM-1*, *VCAM-1*, or *SELP* 3'-UTR-WT) combined with NC or miR-338-5p mimics into 293T cells. **P*< 0.05, ***P*< 0.01, ****P*< 0.001.



Supplemental Figure VIII. The regulatory effects of miR-338-5p on the expression of eNOS and ET-1.

(A-B) Expression of eNOS and ET-1 was detected by ELISA in plasma of control, sham, and DVT animal group (n= 10-15/group). (C-D) eNOS and ET-1 protein levels were determined by ELISA in plasma of DVT animal group and each treatment group (n= 7-12/group). **P*< 0.05, ***P*< 0.01.



Supplemental Figure IX. The role of IL-6 in DVT formation and vascular endothelial function.

(A-C) H&E staining of serial cross sections of IVC from DVT mice treated with IgG1, anti-IL-6 antibody, agomiR-338-5p+IgG1, agomiR-338-5p+anti-IL-6 antibody, antagomiR-338-5p+IgG1, or antagomiR-338-5p+anti-IL-6 antibody at 48 hours (magnification, ×100). Scale bars= 200 μm. (D-F) IL-6 protein levels were determined by ELISA in plasma of DVT animal group and each treatment group (n= 5-12/group). (G) *CCL2*, *CCL3*, *ICAM-1*, *VCAM-1*, and *SELP* mRNA levels were detected by qRT-PCR in PBMCs of DVT mice treated with IgG1 (n= 10) or anti-IL-6 antibody (n= 8). **P*< 0.05, ***P*< 0.01, ****P*< 0.001.

Supplemental Table I. List of polymerase chain reaction (PCR) primer sequences used in miRNA expression analysis.

Gene	Sequence (5'-3')
U6	Forward: CAGCACATATACTAAAATTGGAACG Reverse: ACGAATTTGCGTGTCATCC
miR-338-5p	Forward: CGTTACGCAACAATATCCTGGT Reverse: TATGGTTCTTCACGACTCCTTCAC

Supplemental Table II. List of PCR primer sequences used in mRNA expression analysis.

Gene	Species	Sequence (5'-3')
GAPDH	Human	Forward: ACAACTTTGGTATCGTGGAAGG Reverse: GCCATCACGCCACAGTTTC
IL-6	Human	Forward: CACACAGACAGCCACTCACC Reverse: CACCAGGCAAGTCTCCTCAT
CCL2	Human	Forward: TGCAATCAATGCCCCAGTCA Reverse: GGGTCAGCACAGATCTCCTT
CCL3	Human	Forward: GCTCTCTGCAACCAGTTCTCT Reverse: GGCTTCGCTTGGTTAGGAAGA
ICAM-1	Human	Forward: AGGATGGCACTTTCCCACTG Reverse: GGAGAGCACATTCACGGTCA
VCAM-1	Human	Forward: CCACAGTAAGGCAGGCTGTAA Reverse: GCTGGAACAGGTCATGGTCA
SELP	Human	Forward: CACCAACGAGGCTGAGAACT Reverse: GGCTGACGGACTCTTGATGT
β-actin	Mice	Forward: TCCTTCTTGGGTATGGAATCCTG Reverse: TGCTAGGAGCCAGAGCAGTA
IL-6	Mice	Forward: GGATACCACTCCCAACAGACC Reverse: TTCTGCAAGTGCATCATCGT
CCL2	Mice	Forward: AGTTAACGCCCCACTCACCT Reverse: TGCTGGTGATCCTCTTGTAGC
CCL3	Mice	Forward: GCTTCTCCTACAGCCGGAAG Reverse: AGGTCTCTTTGGAGTCAGCG
ICAM-1	Mice	Forward: CTGGGCTTGGAGACTCAGTG Reverse: CCACACTCTCCGGAAACGAA
VCAM-1	Mice	Forward: CTGGGAAGCTGGAACGAAGT Reverse: GCCAAACACTTGACCGTGAC
SELP	Mice	Forward: GGGCTTCAGGACAATGGACA Reverse: TGGAAGGTGCAGGTTGATCC
TNFα	Mice	Forward: AGCCGATGGGTTGTACCTTG Reverse: ATAGCAAATCGGCTGACGGT
IL-17A	Mice	Forward: CACCGCAATGAAGACCCTGA Reverse: TTCCCTCCGCATTGACACAG

Supplemental Table III. Oligo sequences used in the experiments.

Oligo ID	Sequence (5'-3')
mimic negative control	sense: UUCUCCGAACGUGUCACGUTT antisense: ACGUGACACGUUCGGAGAATT
hsa-miR-338-5p mimics	sense: AACAAUAUCCUGGUGCUGAGUG antisense: CUCAGCACCAGGAUAUUGUUUU
inhibitor negative control	CAGUACUUUUGUGUAGUACAA
hsa-miR-338-5p inhibitor	CACUCAGCACCAGGAUAUUGUU
IL-6-siRNA	sense: GGAGACAUGUAACAAGAGUTT antisense: ACUCUUGUUACAUGUCUCCTT
agomir NC	sense: UUCUCCGAACGUGUCACGUTT antisense: ACGUGACACGUUCGGAGAATT
agomiR-338-5p	sense: AACAAUAUCCUGGUGCUGAGUG antisense: CUCAGCACCAGGAUAUUGUUUU
antagomir NC	CAGUACUUUUGUGUAGUACAA
antagomiR-338-5p	CACUCAGCACCAGGAUAUUGUU

Supplemental Table IV. Thrombus formation in different groups of DVT mice.

Group	Number of mice	Mice number of thrombus formation	Thrombus formation rate (%)
DVT	15	10	67
DVT agomir NC	15	10	67
DVT agomiR-338-5p	15	7	47
DVT antagomir NC	15	10	67
DVT antagomiR-338-5p	15	12	80
DVT+IgG1	15	10	67
DVT+anti-IL-6 antibody	15	8	53
DVT agomiR-338-5p+IgG1	15	7	47
DVT agomiR-338-5p+anti-IL-6 antibody	15	5	33
DVT antagomiR-338-5p+IgG1	15	12	80
DVT antagomiR-338-5p+anti-IL-6 antibody	15	9	60

Supplemental Table V. Data for 30 down-regulated miRNAs filtered in the Chip.

systematic name	C1	C2	C3	C4	C5	C6	D1	D2	D3	D4	D5	D6	active_sequence	chr	Control type	mirbase accession No
hsa-miR-1305	2.890522	2.644196	2.866945	2.967403	2.727855	2.840017	-3.32163	-2.05754	-1.31385	-3.28559	-3.29659	-3.25973	TCTCTCCCATTAGAGTTGA		0	MIMAT0005893
hsa-miR-6717-5p	3.102884	2.69068	3.036068	3.036068	2.856456	2.749102	-3.32163	-3.32188	1.09525	-3.28559	-3.29659	-3.25973	TCTCTACATCCCCACATC		0	MIMAT0025846
hsa-miR-4442	2.519717	2.308948	2.491924	2.232139	2.270366	2.408409	-3.32163	-3.32188	-1.30551	-3.28559	-3.29659	-3.25973	CCTCCCTCTTGTCCG		0	MIMAT0018960
hsa-miR-4653-3p	2.557016	2.396131	2.347332	2.361599	2.016503	2.379267	-3.32163	-3.32188	-1.23594	-3.28559	-3.29659	-3.25973	TCTCCAAGCAACCCTT		0	MIMAT0019719
hsa-miR-4419a	2.715025	2.169307	2.133044	2.176626	2.032571	2.151727	-3.32163	-3.32188	0.128117	-3.28559	-3.29659	-3.25973	TGCAGTCTCCTCCCT		0	MIMAT0018931
hsa-miR-564	3.283139	2.460638	2.532001	2.396131	2.491924	2.58784	-3.32163	0.948874	-1.72766	-3.28559	-3.29659	-3.25973	GCCTGCTGACACCGT		0	MIMAT0003228
hsa-miR-483-5p	3.317903	2.250299	2.232139	1.747687	2.151727	2.075307	-3.32163	-1.25142	1.859515	-3.28559	-3.29659	-3.25973	CTCCCTTCTTTCCTC		0	MIMAT0004761
hsa-miR-4788	2.331065	2.049457	2.106463	2.106463	1.798728	2.123388	-1.67023	-0.63613	0.205429	-3.28559	-3.29659	-3.25973	GCCTCCCTTAGCTGG		0	MIMAT0019958
hsa-miR-5684	1.261394	1.025808	1.820038	1.16116	1.453198	1.478231	-3.32163	-3.32188	-2.0638	-3.28559	-3.29659	-3.25973	CTGTTGCTCAGGCTAG		0	MIMAT0022473
hsa-miR-650	2.032571	1.380212	2.075307	1.392273	0.005068	1.722659	-2.0638	-2.11512	-1.68486	-3.28559	-0.6177	-3.25973	GTCCTGAGAGCGCTGC		0	MIMAT0003320
hsa-miR-6891-5p	2.615863	1.131285	1.058549	1.193515	1.261998	1.641266	-3.32163	0.834653	-0.2856	-3.28559	-3.29659	-3.25973	CCCCTCATCCCCC		0	MIMAT0027682
hsa-miR-4713-3p	3.658131	3.219048	3.235708	3.544785	2.989471	3.16269	1.418389	1.620391	1.958252	-3.28559	0.197783	-3.25973	TTCTCCCACTGTCTGG		0	MIMAT0019821
hsa-miR-6769b-5p	2.097667	3.315963	0.192071	2.169307	1.058549	3.289554	-3.32163	-3.31385	-3.32138	-3.28559	-3.29659	-3.25973	GCACTTCTCCTCCCC		0	MIMAT0027620
hsa-miR-6740-5p	4.015547	3.437881	3.584511	3.707074	3.544785	3.637127	1.747687	2.123388	2.232139	1.213376	2.097667	-3.25973	TCTCCTCTCTCCATCCC		0	MIMAT0027381
hsa-miR-6126	2.450542	2.532001	2.331065	2.379267	2.185673	2.48021	0.928946	-1.22525	1.732256	-3.28559	0.140876	1.317771	TCTCCGCCGGGC		0	MIMAT0024599
hsa-miR-513c-5p	0.834653	0.948874	0.250975	0.197783	-0.65314	1.115497	0.005068	-2.21119	-2.14442	-3.28559	-0.66784	-3.25973	ATAAACGACACCTCCTTGA	chrX	0	MIMAT0005789
hsa-miR-6850-5p	2.049457	0.205429	1.747687	-1.24639	3.296593	3.289554	-3.32163	-3.32188	-3.34287	-3.28559	-3.29659	-3.25973	CGCCCCGGCCA		0	MIMAT0027600
hsa-miR-338-5p	0.861888	0.140876	0.993548	0.176005	0.050273	0.050273	-2.1352	-2.14138	-1.71401	-3.28559	-3.29659	0.897482	CACTCAGCACCAGGA		0	MIMAT0004701
hsa-miR-4449	1.180867	1.115497	0.168898	1.144027	1.180867	0.250975	-0.66917	-0.29264	0.140876	-3.28559	-0.2856	-3.25973	TGCCTCGCGCAGC		0	MIMAT0018968
hsa-miR-6724-5p	2.218914	2.232139	1.392273	1.765325	1.581486	1.131285	0.240207	1.006592	0.948874	0.215132	0.993548	-3.25973	CCCCACGCCCG		0	MIMAT0025856
hsa-miR-6879-5p	3.915201	3.658131	3.812723	3.742235	3.707074	3.780678	2.29194	2.519717	2.749102	2.460638	2.58784	2.424246	CTCTCCCACCTTCCC		0	MIMAT0027658
hsa-miR-6780b-5p	3.683952	3.283139	3.482658	3.437881	3.437881	3.437881	2.097667	2.460638	2.491924	2.097667	2.408409	1.732256	TCTTCCCTGCCAAGC		0	MIMAT0027572
hsa-miR-874-3p	2.199508	2.840017	2.69068	2.727855	2.644196	2.715025	0.948874	2.106463	1.180867	1.418389	1.581486	1.115497	TCGGTCCCTCGGG	chr5	0	MIMAT0004911
hsa-miR-6820-5p	5.146708	5.105475	5.017525	4.920903	4.920903	5.35999	3.848797	3.402584	4.132329	3.780678	3.707074	4.159892	TGACCCCAGCTCTGC		0	MIMAT0027540
hsa-miR-150-5p	10.77513	10.77513	10.77513	10.77513	9.949985	10.52436	9.41637	9.949985	8.878604	9.41637	9.949985	8.670342	CACTGGTACAAGGGTTGG	chr19	0	MIMAT0000451
hsa-miR-6127	5.439865	4.756314	4.920903	4.860059	4.860059	4.827461	3.525265	3.729768	3.986414	3.848797	4.100742	3.848797	CCTCCCACCCACTC		0	MIMAT0024610
hsa-miR-6875-5p	5.105475	4.423265	4.756314	4.535265	4.470753	4.617086	3.463085	3.584511	3.615922	3.463085	3.683952	3.565681	TCTCCTGTCCTGGGT		0	MIMAT0027650
hsa-miR-1233-5p	5.273259	4.860059	4.893096	4.72706	4.689545	4.920903	3.729768	3.463085	4.06103	3.729768	3.812723	4.036973	TGCCGTGCCCTGG	chr15	0	MIMAT0022943
hsa-miR-342-5p	3.812723	3.637127	3.742235	4.100742	3.634786	3.05275	2.331065	3.336585	1.703047	2.396131	2.989471	1.782291	TCAATCACAGATAGCACCC		0	MIMAT0004694
hsa-miR-324-3p	6.365108	6.310584	6.456261	6.66172	6.409631	6.365108	5.146708	5.273259	5.56788	5.146708	5.638115	5.439865	CCAGCAGCACCTGGGG	chr17	0	MIMAT0000762