Supplementary File 1

Supplementary methods

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Hemolysis of serum specimens was tested to remove potential contaminants. We routinely assessed the degree of overall RNA integrity to analyze RNA quality. Samples exhibiting an RNA integrity number (RIN) value greater than 7 as determined on an Agilent 2100 Bioanalyzer were included in the analysis. miRNA was isolated from serum samples with a miRNeasy Mini Kit (Qiagen). A total of 500 µl of serum was used for each experiment. For the detection of miR-18a-5p in serum samples, a synthetic C. elegans miRNA (cel-miRNA-39) was used as an internal control due to the lack of universal endogenous controls. miR-18a-5p primers were obtained from Takara Bio (sequences protected by a patent). Other primers were obtained from Sangon Biotech, and detailed primer sequence information is listed in Supplementary Table 1.

Enriched small RNAs from serum were reverse transcribed with a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). The expression of selected miRNAs, cel-miR-39 (for normalization of serum) and U6 (for normalization of cultured cells) was tested with a TaqMan miRNA assay (Applied Biosystems).

Total RNA from cells was extracted using TRIzol reagent (Invitrogen, USA). The RNA concentration and integrity were determined by spectrophotometry and standard RNA gel electrophoresis, respectively. RNA was reverse transcribed into cDNA using a Primer-Script One-Step RT-PCR kit (TaKaRa, Dalian, China). The cDNA template was amplified by real-time PCR using a SYBR Premix DimerEraser kit (TaKaRa). Real-time PCR was performed using specific primers (Supplementary Table 1) for GAPDH, TSP1, IGF1, LIF, CCND2, NACC1 and MMP1 obtaining from Sangon Biotech. CTGF primers were obtained from Takara Bio (sequences protected by a patent). The mean cycle threshold (CT) value for each sample was analyzed using the $2^{-\Delta\Delta CT}$ method. All the reactions were performed in triplicate.

Cell proliferation assay

Cell viability was measured using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). After transfection for 48 hours, HUVECs were seeded into 96-well plates at a density of 1×10^3 cells per well in 100 µl of medium. Subsequently, 10 µl of CCK-8 solution with 90 µl medium was added to each well at the indicated time points. After incubation at 37°C for 90 min, the absorbance at 450 nm was measured with a plate reader. The growth curves were examined to determine the growth rates.

Transwell assay

HUVECs were seeded into the upper chamber of Transwell® cell culture inserts with an 8 µm pore size (Costar-Corning, USA) in serum-free medium. The lower chamber was filled with MEM-α supplemented with 10% FBS. After an incubation period of 48 h, the cells on the upper surface of the membrane were removed with a cotton swab, while cells in the bottom of the chamber were fixed with 70% ethanol and stained with 0.5% crystal violet solution for 30 min at room temperature. A light microscope was used to count the number of migrated cells (4 random fields per chamber).

Wound healing assay

HUVECs were plated in 6-well plates at a density of 2×10^6 cells per well. After 24 h, wounds were generated using sterilized pipette tips, and the medium was substituted with serum-free medium. Images of the plates in the same field of view were observed and captured 48 h later.

In vitro tube formation assay

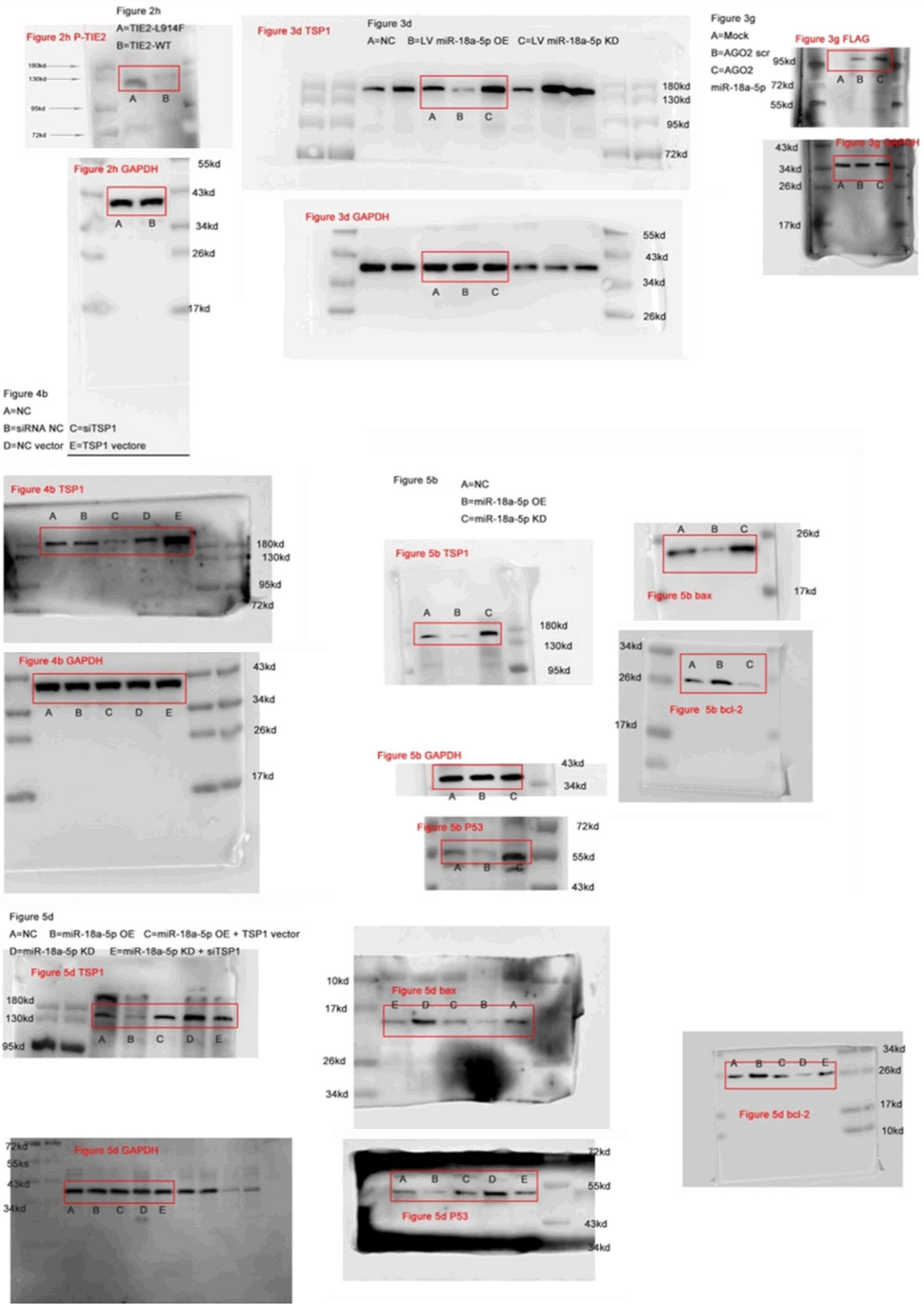
In vitro tube formation assays were performed in 96-well plates coated with 50 μl of Matrigel Basement Membrane Matrix, Growth Factor Reduced (Corning, 354230), which was heated at 37°C for 30 min to allow gel formation. Then, 2×10^4 HUVECs subjected to various transfections in MEM-α containing 2% FBS were seeded onto the Matrigel and incubated at 37°C for 8 h. The formation of tubes and tube-like structures was captured 8 h later using a Nikon Eclipse 80i microscope (Nikon, Japan).

RNA immunoprecipitation (RIP)

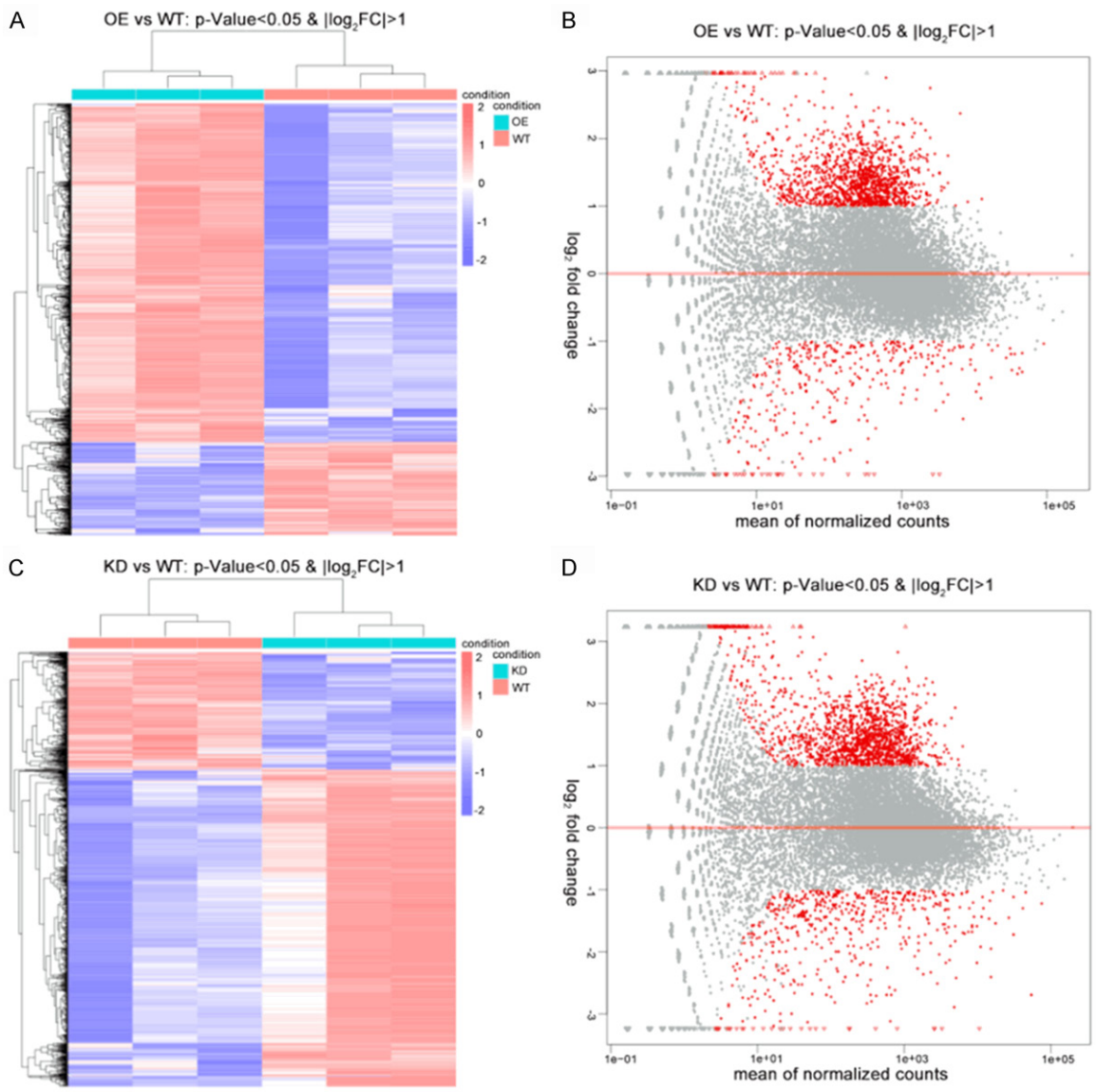
HUVECs (2×10^6) were seeded onto 10 cm tissue culture plates and transfected with 4 μg of FLAG-AGO2 and 25 nM scr/mimic miR-18a-5p using Lipofectamine 2000 (Life). HUVECs were transfected with pBSKS (an empty plasmid) as a negative control. After transfection for 48 hours, cells were washed with ice-cold 1× PBS, scraped, and transferred to a 1.5 ml tube, followed by centrifugation at 200×g for 2 min. The cells were then resuspended in 200 μl of cold resuspension buffer (20 mM Tris (pH = 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA) containing 1 U/μl RNasin Plus (Promega) and lysed by the addition of 800 μl of cold lysis buffer (1% Triton X-100, 20 mM Tris (pH = 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethyl-sulfonyl fluoride (PMSF, Sigma), 1X complete EDTA-free Protease Inhibitor cocktail (Roche) and incubation on ice for 10-30 min. After centrifugation (10,000×g for 10 min at 4°C), 10 μl of RQ1 DNase (Promega) was added to the supernatant. IP of FLAG-AGO2 was performed with anti-FLAG M2 mouse antibody (Sigma, F3165) for 3 h at 4°C with rotation. After undergoing five washes with lysis buffer, 10% of the sample beads were subjected to protein extraction and WB by adding LDS sample buffer and DTT and heating the samples at 70°C for 20 min. The remaining 90% of the sample beads were incubated with RQ1 DNase for 30 min for later RNA extraction with TRIzol (Invitrogen, USA).

Establishment of an animal model of VM

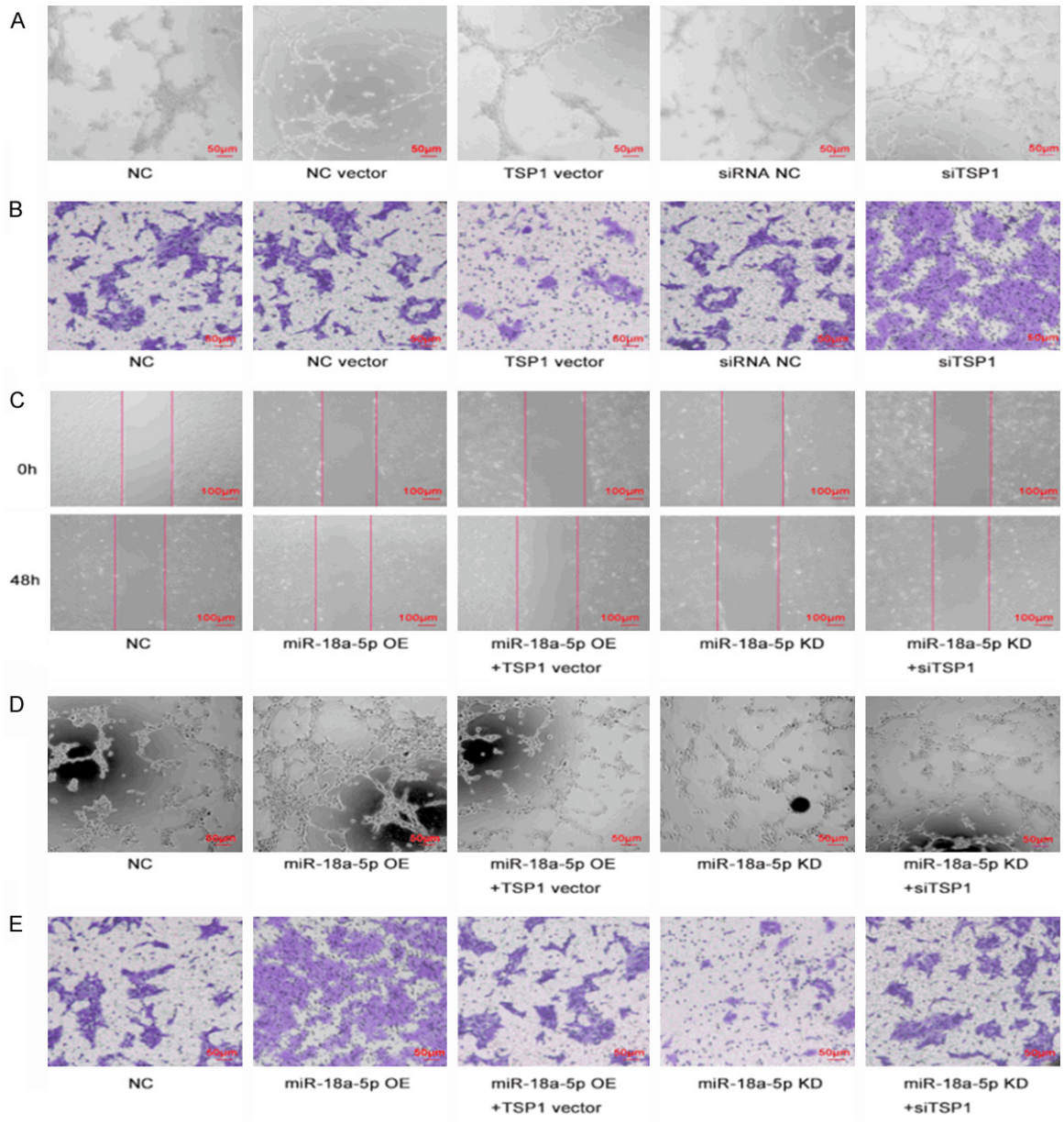
Six-week-old athymic BALB/c nu/nu male mice (purchased from ProMedican Pharmaceutical Co.) were used to establish the VM model. HUVECs (5×10^6) stable cell lines which expressing TIE2 protein (WT or L914F mutation) were subcutaneously inoculated into either side of the flank area in an equivalent volume of Matrigel (n = 3 for each group). The lesions were harvested 2 weeks after inoculation and subjected to HE staining. For the miR-18a-5p treatment experiment, HUVECs with the TIE2-L914F mutation were transfected with miR-18a-5p mimic, inhibitor or vehicle for 72 h before subcutaneous injection into mice as previously described (n = 6 per group). The lesion area was measured with Vernier calipers every 3 days, and the final lesions were harvested at 18 days after inoculation. The weight of lesions and the vessel structures were observed after HE staining. All animal experiments were performed in the animal laboratory center of the Ninth People's Hospital, Shanghai Jiaotong University School of Medicine (Shanghai, China), and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication number 85-23, revised 1996). The study protocol was approved by the Animal Care and Use Committee of the Ninth People's Hospital (SH9H-2019-A486-1).



Supplementary Figure 1. Original, full-length gel and blot images.

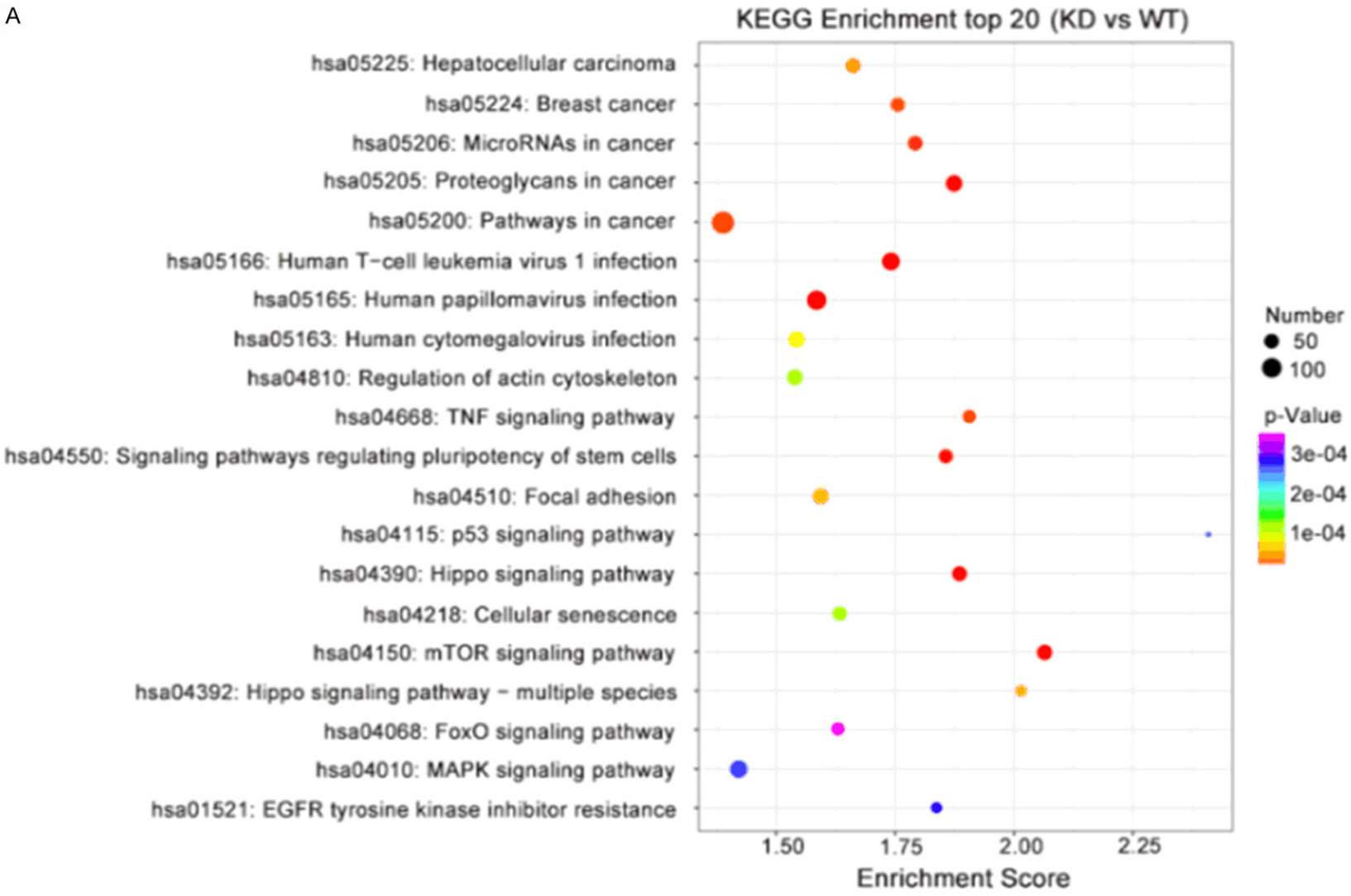


Supplementary Figure 2. A, C. A heatmap showing the genes differentially expressed between the miR-18-5p changed group and the control group as indicated via RNA-seq is shown. B, D. Volcano plot showing all differentially expressed genes between the miR-18-5p changed group and the control group as indicated via RNA-seq is shown.

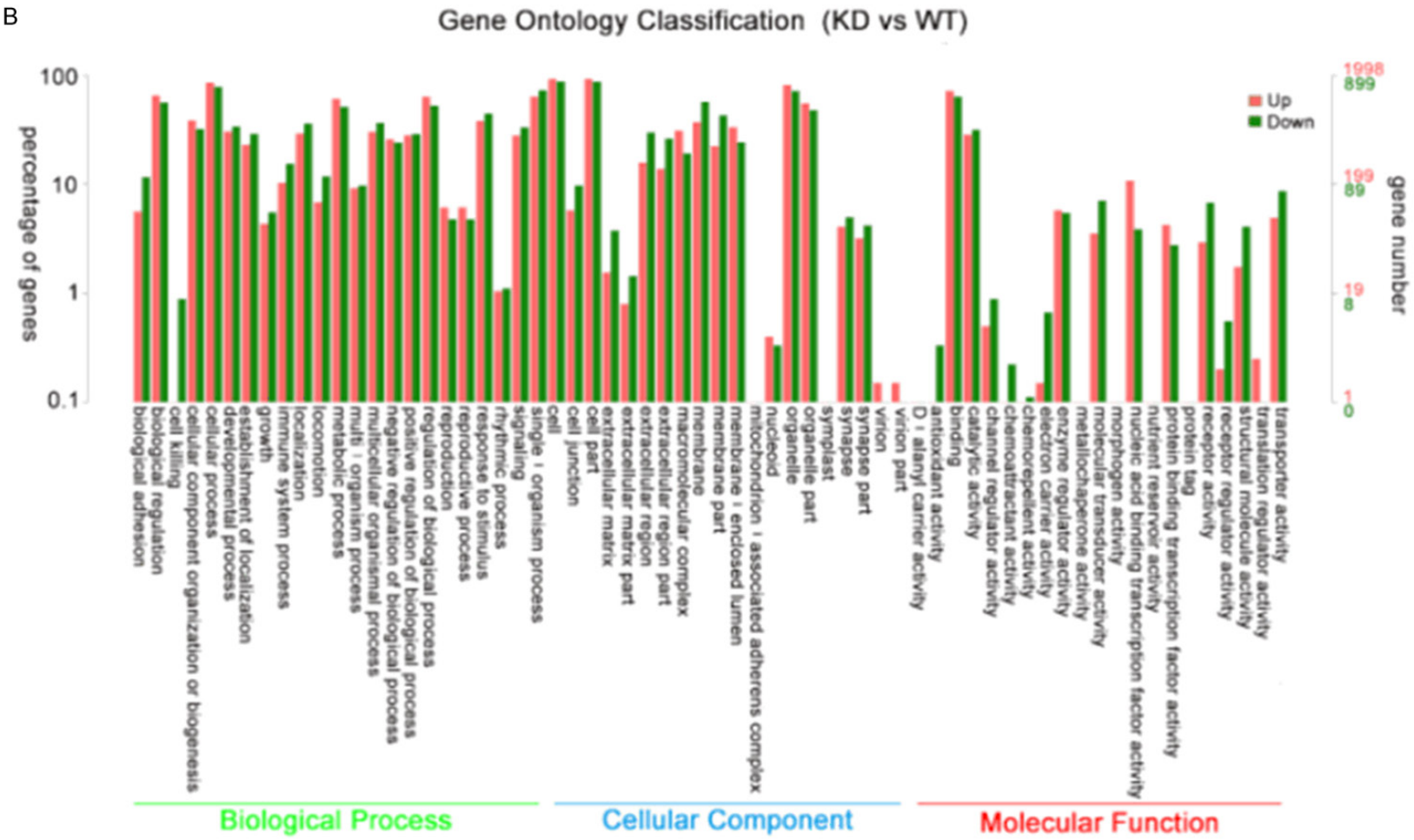


Supplementary Figure 3. (A, B) Angiogenesis and migration of HUVECs transfected with NC, NC vector, TSP1 vector, siRNA NC or siTSP1 were evaluated by tube formation (A) and transwell assays (B), respectively. (C-E) Invasion, angiogenesis and migration of HUVECs transfected with NC, miR-18a-5p OE, miR-18a-5p OE + TSP1 vector, miR-18a-5p KD and miR-18a-5p KD + siTSP1 were evaluated by wound healing (C), tube formation (D) and transwell assays (E), respectively.

A


KEGG Enrichment top 20 (KD vs WT)
hsa05225: Hepatocellular carcinoma
hsa05224: Breast cancer
hsa05206: MicroRNAs in cancer
hsa05205: Proteoglycans in cancer
hsa05200: Pathways in cancer
hsa05166: Human T−cell leukemia virus 1 infection
hsa05165: Human papillomavirus infection
hsa05163: Human cytomegalovirus infection
hsa04810: Regulation of actin cytoskeleton
hsa04668: TNF signaling pathway
hsa04550: Signaling pathways regulating pluripotency of stem cells
hsa04510: Focal adhesion
hsa04115: p53 signaling pathway
hsa04390: Hippo signaling pathway
hsa04218: Cellular senescence
hsa04150: mTOR signaling pathway
hsa04392: Hippo signaling pathway − multiple species
hsa04068: FoxO signaling pathway
hsa04010: MAPK signaling pathway
hsa01521: EGFR tyrosine kinase inhibitor resistance
1.50
1.75
2.00
2.25
Enrichment Score
Number
50
100
p-Value
3e-04
2e-04
1e-04

B


Gene Ontology Classification (KD vs WT)
percentage of genes
100
10
1
0.1
gene number
1998
899
199
89
19
8
1
0
Up
Down
biological adhesion
biological regulation
cell killing
cellular component organization or biogenesis
cellular process
developmental process
establishment of localization
growth
immune system process
localization
locomotion
metabolic process
multi-organism process
multicellular organismal process
negative regulation of biological process
positive regulation of biological process
regulation of biological process
reproduction
reproductive process
response to stimulus
rhythmic process
signaling
single-organism process
cell
cell junction
cell part
extracellular matrix
extracellular matrix part
extracellular region
extracellular region part
macromolecular complex
membrane
membrane part
membrane-enclosed lumen
mitochondrion-associated adherens complex
nucleoid
organelle
organelle part
symplast
synapse
synapse part
virion
virion part
D-alanyl carrier activity
antioxidant activity
binding
catalytic activity
channel regulator activity
chemoattractant activity
chemorepellent activity
electron carrier activity
enzyme regulator activity
metallochaperone activity
molecular transducer activity
morphogen activity
nucleic acid binding transcription factor activity
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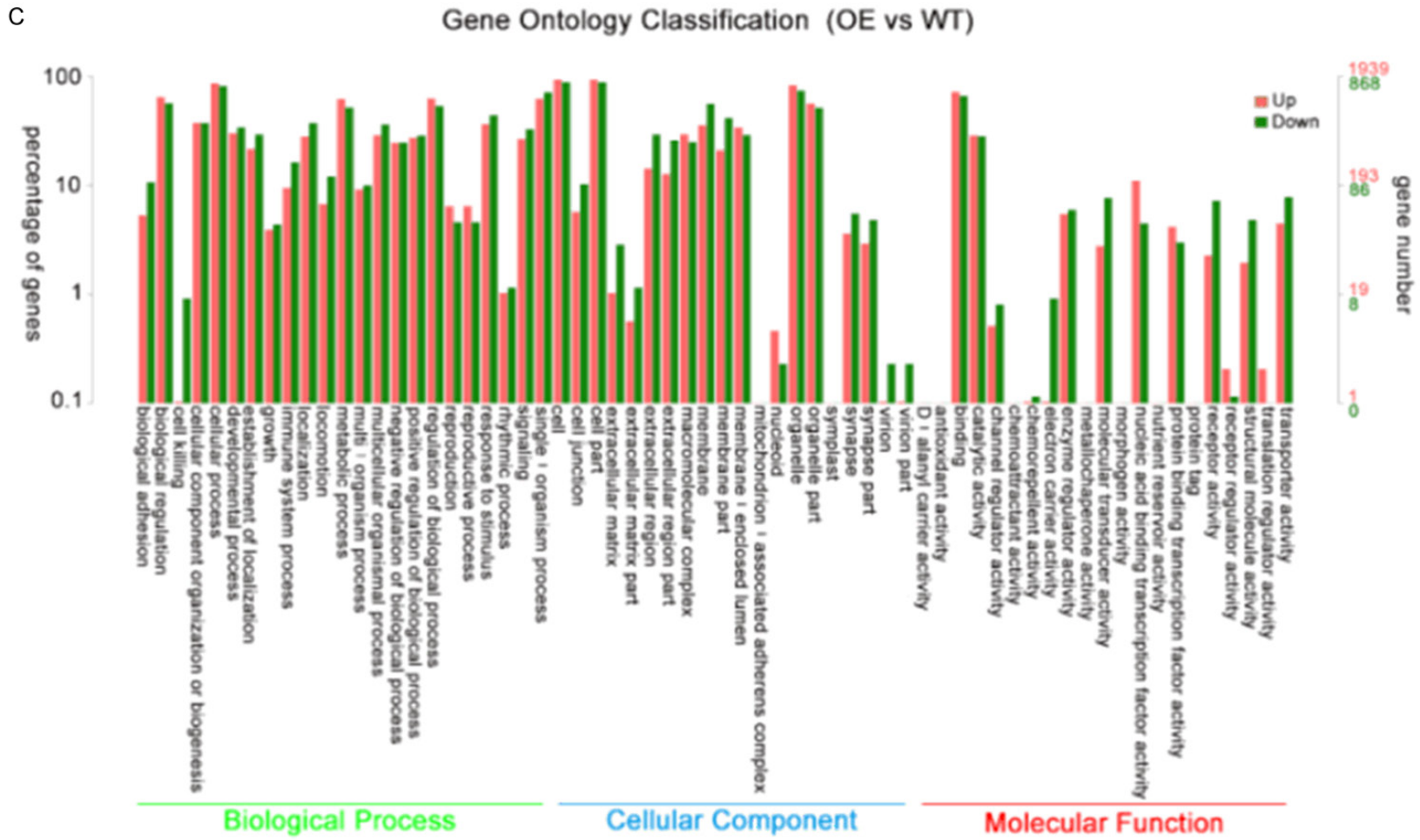
receptor activity
receptor regulator activity
structural molecule activity
translation regulator activity
transporter activity
Biological Process
Cellular Component
Molecular Function



Supplementary Figure 4. (A) KEGG analysis was used to identify pathway terms enriched in the dysregulated mRNAs in HUVECs with miR-18a-5p KD vs WT. (B, C) GO term enrichment analysis of the biological process (BP), cellular component (CC) and molecular function (MF) categories for the dysregulated mRNAs (B: KD vs WT, C: OE vs WT).

Supplementary Table 1. Primers of genes used for this study

Primer	Sequences
Cel-miR-39	Forward Primer 5'-CAGAGTCACCG GGTGTAAAT-3'
	Reverse Primer 5'-CCAGTGC GTGTCGTGGAGTC-3'
U6	Forward Primer 5'-CTCGCTTCGGCAGCACATATACT-3'
	Reverse Primer 5'-ATTTGCGTGTCATCCTTGCGCA-3'
TSP-1	Forward Primer 5'- GCCATCCGCACTAACTACATT-3'
	Reverse Primer 5'-TCCGTTGTGATAGCATAGGGG-3'
IGF1	Forward Primer 5'-GCTCTTCAGTTCGTGTGTGGA-3'
	Reverse Primer 5'-GCCTCCTTAGATCACAGCTCC-3'
NACC1	Forward Primer 5'-CTCTCCCGGCTGAACTTATCA-3'
	Reverse Primer 5'-AGCGTGTTCCGGTCAAAGAA-3'
LIF	Forward Primer 5'-GCATCAACTCCGCAGCTTAG-3'
	Reverse Primer 5'-CTGAACGCCATAGCCAGGTCT-3'
CCND2	Forward Primer 5'-ACCTTCCGCAGTGCTCCTA-3'
	Reverse Primer 5'-CCCAGCCAAGAAACGGTCC-3'
MMP1	Forward Primer 5'-AAAATTACACGCCAGATTTGCC-3'
	Reverse Primer 5'-GGTGTGACATTACTCCAGAGTTG-3'
GAPDH	Forward Primer 5'- GGAGCGAGATCCCTCCAAAAT-3'
	Reverse Primer 5'- GGCTGTTGTCATACTTCTCATGG-3'