Supplementary data for

N6-methyladenosine induced miR-143-3p promotes the brain metastasis of lung cancer via regulation of VASH1

Figure S1

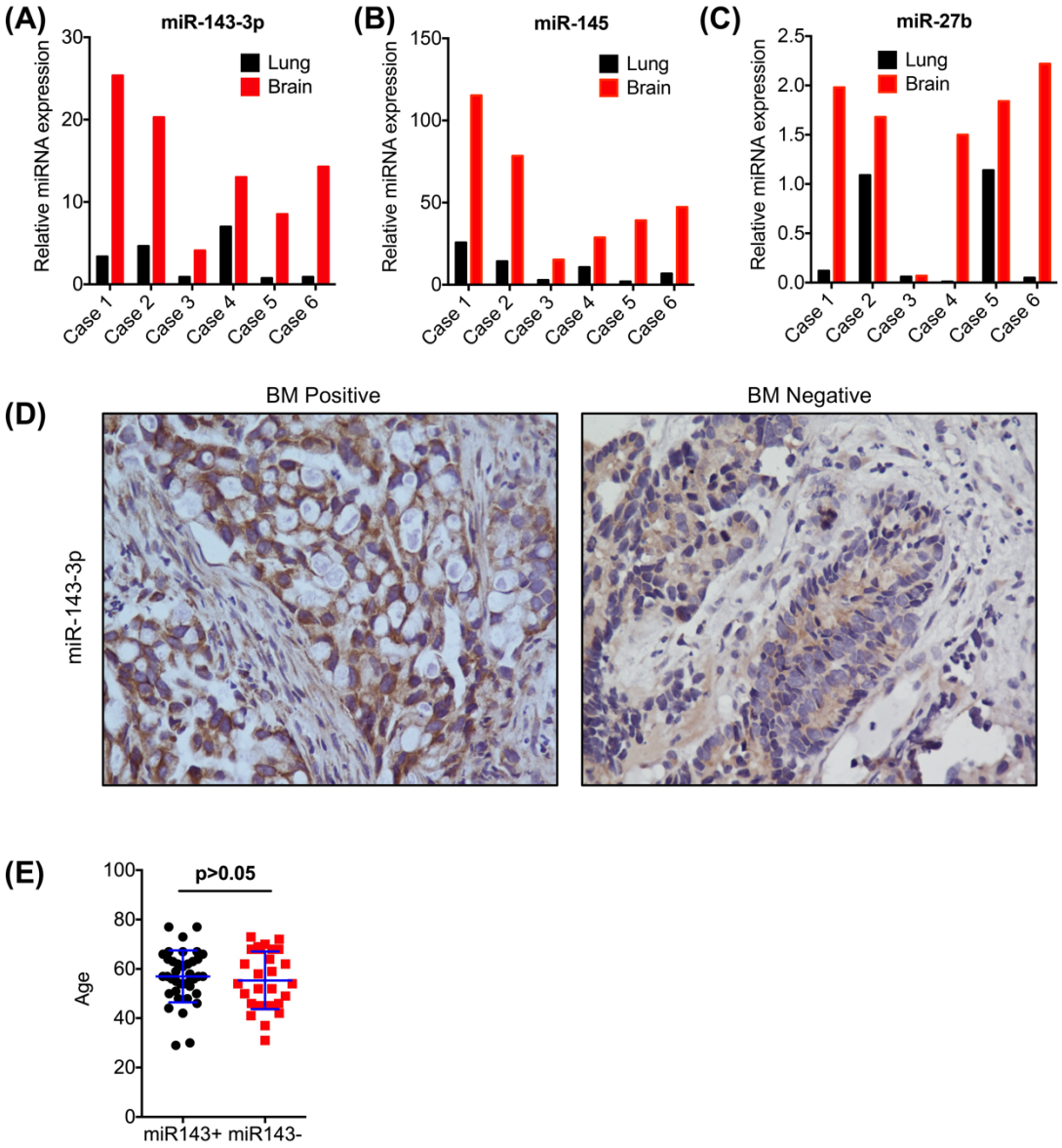

Figure S1 miR-143-3p is correlated with the BM and progression of lung cancer.

(A~C) The miRNA expression profiles in 6 matched pairs of primary lung cancer and BM tissues were analysed by qRT-PCR;

(D) The expression of miR-143-3p in lung cancer patients with or without BM was measured by ISH;

(E) The age of miR-143-3p- or miR-143-3p+ lung cancer patients;

Figure S2

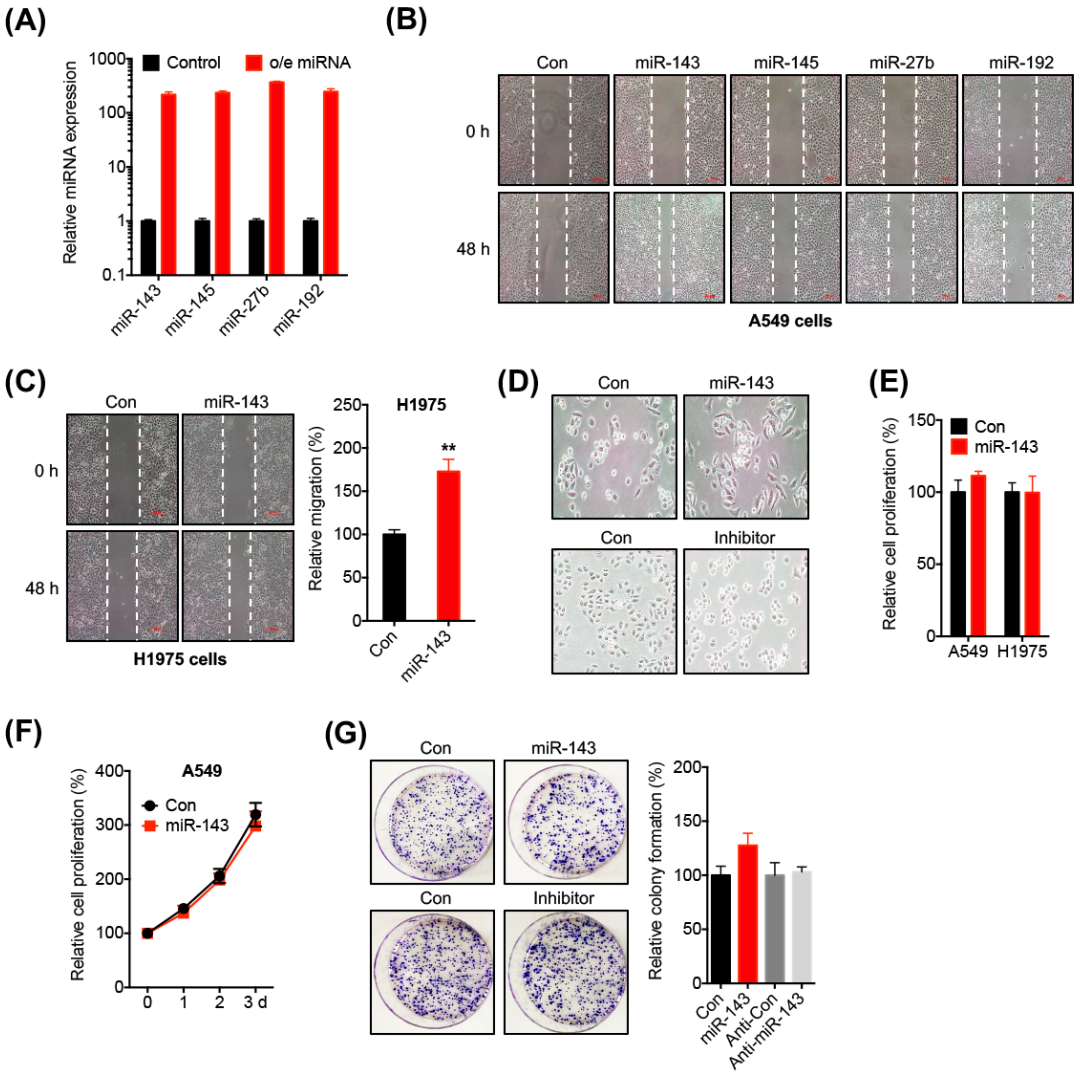

Figure S2 The effects of miRNAs on the malignancy of lung cancer cells.

(A) A549 cells were transfected with scramble RNA (Con) or miRNA-143-3p construct for 24 h, the expression of miRNAs was checked by qRT-PCR;

(B) The relative migration of A549 cells transfected with miRNAs was measured by wound healing assay;

(C) H1975 cells were transfected with or without miR-143-3p for 48 h, the cell migration was measured by wound healing assay (*left*) and quantitatively analyzed (*right*);

(D) A549 cells were transfected with scramble RNA (Con), miRNA-143-3p, siRNA control, or miR-143-3p inhibitor for 48 h, the cell morphological changes were recorded by a phase contrast microscope;

(E) Cells were transfected with scramble RNA (Con) or miRNA-143 for 24 h, the cell proliferation was tested by CCK-8 kit;

(F) A549 cells were transfected with scramble RNA (Con) or miRNA-143 for the indicated time periods, the cell proliferation was tested by CCK-8 kit;

(G) A549 cells were transfected with scramble RNA (Con), miRNA-143, siRNA control, or miR-143-3p inhibitor for 24 h and then split and cultured in fresh medium for the next 15 days. The colonies were fixed with methanol/glacial acetic acid (7:1) and stained with 0.1% of crystal violet.

Data are presented as the mean ± SD from three independent experiments. *$p<0.05$, ** $p<0.01$ compared with control.

Figure S3

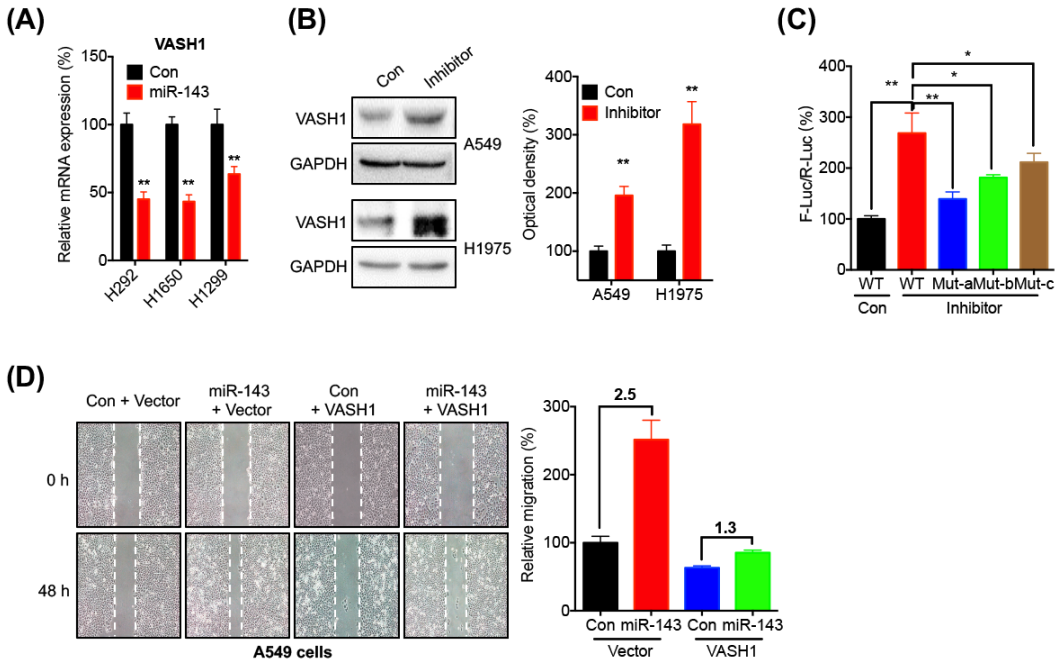

Figure S3 VASH1 mediates miR143 induced cell dissemination and angiogenesis

(A) Lung cancer cells were transfected with control or miR-143-3p for 24 h, the expression of VASH1 was measured by qRT-PCR;

(B) A549 or H1975 cells were transfected with siRNA control or miR-143-3p inhibitor for 24 h, the expression of VASH1 was measured by western blot analysis (*left*) and quantitatively analyzed (*right*);

(C) H1299 cells were co-transfected with siRNA control, miR-143-3p inhibitor, pmiR-GLO-WT, and pmiR-GLO-Mut-a/b/c for 24 h, the value of firefly luciferase (F-luc) was normalized to that of the Renilla luciferase (R-luc);

(D) A549 cells were co-transfected with control, miR-143-3p, vector, or VASH1 construct for 48 h, the cell migration was measured by wound healing assay (*left*) and quantitatively analyzed (*right*).

Data are presented as the mean ± SD from three independent experiments. *p<0.05, ** p<0.01 compared with control.

Figure S4

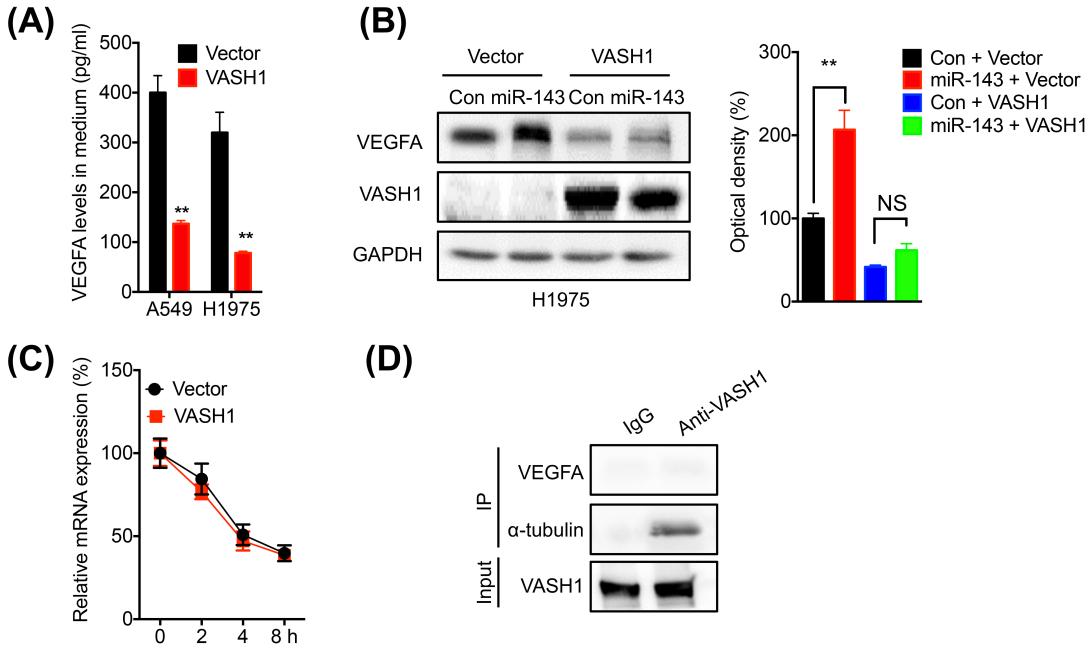

Figure S4 VASH1 mediates miR-143-3p induced angiogenesis via destabilization of VEGFA

(A) A549 or H1975 cells were transfected with vector control or VASH1 construct for 24 h, the expression of VEGFA was measured by ELISA;

(B) H1975 cells were co-transfected with control scramble RNA, miR-143-3p, vector control or VASH1 construct for 24 h, the expression of VEGFA was measured by western blot analysis (*left*) and quantitatively analyzed (*right*);

(C) A549 cells transfected with vector control or VASH1 construct for 24 h were pre-treated with Act-D for another 90 min, then VEGFA mRNA was analyzed at indicated times;

(D) The interaction between VEGFA and VASH1 was checked by co-immunoprecipitation (co-IP) in A549 cells, α-tubulin was used as the positive control for co-IP (1-3).

Data are presented as the mean ± SD from three independent experiments. ** $p<0.01$ compared with control. NS, no significant.

Figure S5

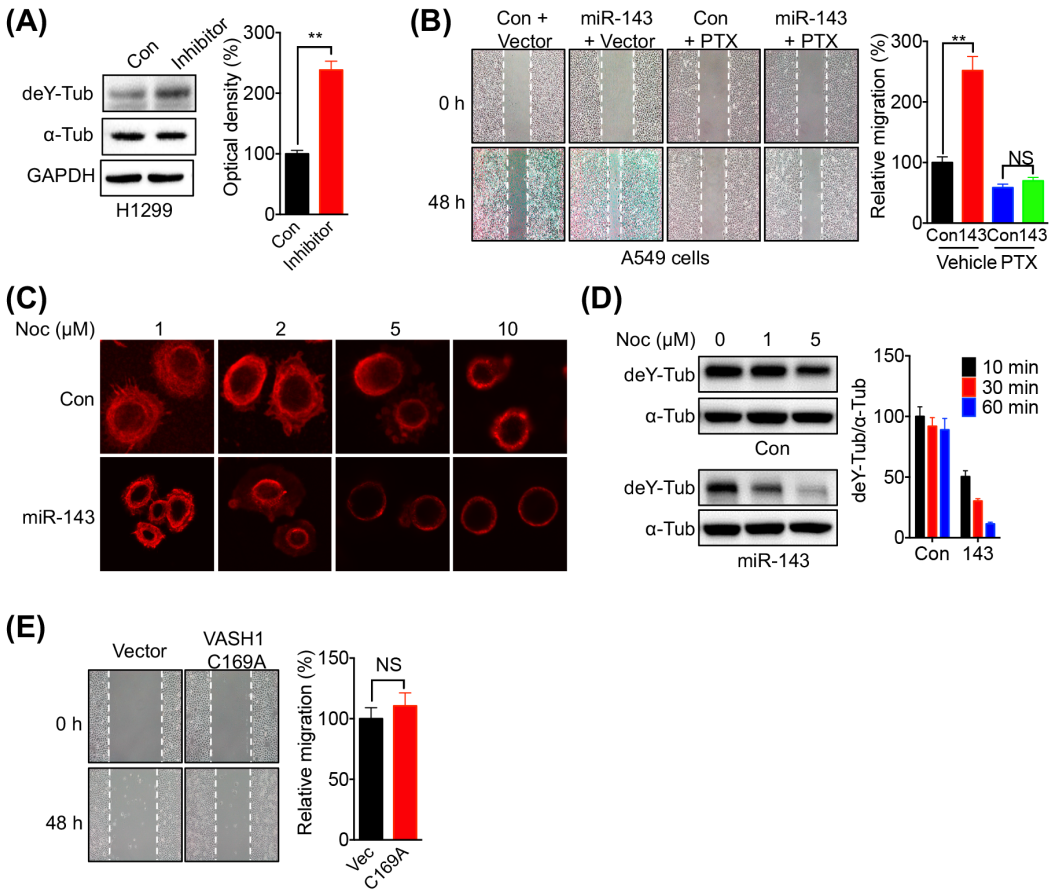

Figure S5 VASH1 mediates miR-143-3p induced reprogramming of microtubules

(A) H1299 cells were transfected with siRNA control or miR-143-3p inhibitor for 24 h, the expression of deY-Tub and Tub was measured by western blot analysis (*left*) and quantitatively analyzed (*right*);

(B) The control and miR-143-3p stable A549 cells were treated with or without PTX (2 μM) for 2 h and then further cultured for 48 h, the wound healing was recorded (*left*) and quantitatively analyzed (*right*);

(C) Immunofluorescence staining of MTs in control and miR-143-3p stable A549 cells treated with different concentrations of nocodazole as indicated for 10 minutes;

(D) The expression of deY-Tub and tubulin in control and miR-143-3p stable A549 cells treated with increasing concentration of nocodazole for 10 min and followed by a washout and incubation in fresh culture medium at 37°C for 10. The levels of deY-tubulin and total tubulin were measured by western blot analysis (*left*) and quantitatively analyzed (*right*).

(E) A549 cells were transfected with vector or VASH1-C169A construct for 48 h, the wound healing was recorded (*left*) and quantitatively analyzed (*right*).

Data are presented as the mean ± SD from three independent experiments. *$p<0.05$, ** $p<0.01$ compared with control.

Figure S6

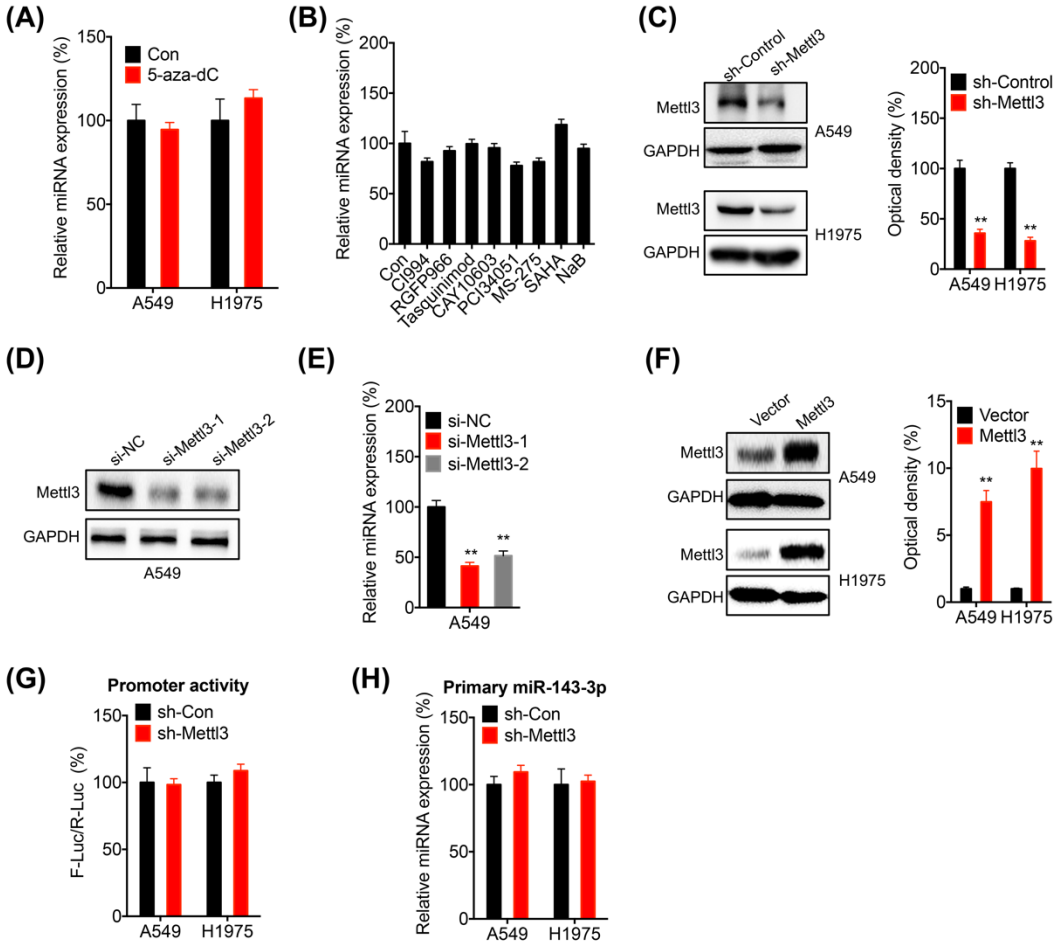

Figure S6 m^{6}A regulates the expression of miR-143-3p in lung cancer cells

(A) Cells were treated with or without 5 μM 5-aza-dC for 4 days, and miR-143-3p expression was measured by qRT-PCR;

(B) A549 cells were treated with specific inhibitors of HDAC1 (CI994, 10 μM), HDAC3 (GFRP966, 10 μM), HDAC4 (Tasquinimod, 10 μM), HDAC6 (CAY1215, 4 μM), HDAC8 (PCI34051, 5 μM), HDAC 1 and 3 (MS-275, 10 μM), SAHA (2 μM), or NaB (2 mM) for 24 h, the expression of miR-143-3p was checked by qRT-PCR;

(C) Cells were transfected with sh-Control or sh-Mettl3 for 24 h, the expression of Mettl3 was measured by western blot analysis (*left*) and quantitatively analyzed (*right*);

(D&E) A549 cells were transfected with si-NC or siRNAs for Mettl3 for 24 h, the expression of Mettl3 was measured by western blot analysis (D), and the expression of miR-143-3p was detected by qRT-PCR (E);

(F) Cells were transfected with vector control or Mettl3 construct for 24 h, the expression of Mettl3 was measured by western blot analysis (*left*) and quantitatively analyzed (*right*);

(G) A549 cells were transfected with sh-Control or sh-Mettl3 for 24 h, the promoter activity of miR-143-3p was measured by luciferase assay;

(H) Cells were transfected with vector control or Mettl3 construct for 24 h, the expression of primary miR-143-3p was measured by qRT-PCR.

Data are presented as the mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$ compared with control.

Related to Figure 6

Figure S7

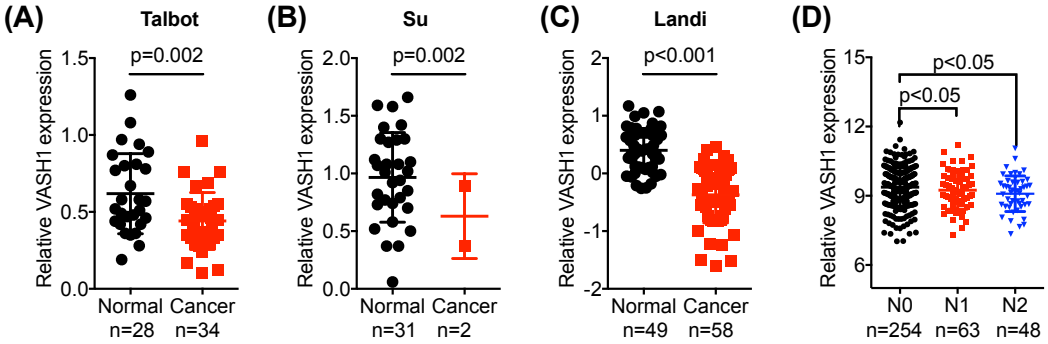

Figure S7 The miR-143-3p/VASH axis and *in vivo* BM of lung cancer

(A~C) Expression of VASH1 in NSCLC (cancer) and adjacent normal tissues from three Oncomine datasets: Talbot (A); Su (B); Landi (C);

(D) VASH1 expression in lung cancers of N0 (n=254), N1 (n=63), and N2 (n=48) stages from TCGA database.

Table S1 Clinical characteristics of 6 lung cancer patients with BM

Case	Gender	Age	Smoke	Pathology	EGFR
1	M	61	Yes	AD	WT
2	M	72	Yes	AD	WT
3	F	29	No	AD	WT
4	F	54	No	AD	WT
5	M	57	Yes	SQ	WT
6	M	49	No	AD	21L858 mut

Table S2 Clinical characteristics of lung cancer patients with or without BM.

Characteristics	n	miR-143-3p+	miR-143-3p-	p value
Gender				
Male	41	24	17	0.839
Female	25	14	11	
Age				
≤50	20	9	11	0.172
＞50	46	29	17	
T Stage*				
I/II	31	17	14	0.570
III/IV	29	18	11	
N Stage**				
0/1	10	8	2	0.116
2/3	49	26	23	
BM				
Negative	32	13	19	**0.006**
Positive	34	25	9	

* 6 samples had no T stage information

** 7 samples had no T stage information

Table S3 The potential targets of miR-143-3p predicted by five different web-based databases

Gene	miRanda	miRDB	miRWalk	RNA22	Targetscan	SUM
VASH1	1	1	1	1	1	5
ZIM3	1	1	1	1	1	5
USP54	1	1	1	1	1	5
WASF3	1	1	1	1	1	5
GABRA4	1	1	1	1	1	5
GABRB3	1	1	1	1	1	5
TMOD2	1	1	1	1	1	5
HK2	1	1	1	1	1	5
IGFBP5	1	1	1	1	1	5
SH2D4B	1	1	1	1	1	5
FLJ40142	1	1	1	1	1	5
MYO5A	1	1	1	1	1	5
CRLF3	1	1	1	1	1	5
SLC38A2	1	1	1	1	1	5
OTUD4	1	1	1	1	1	5
ELMOD1	1	1	1	1	1	5
PAG1	1	1	1	1	1	5
RAB22A	1	1	1	1	1	5
DIP2B	1	1	1	1	1	5
SLC16A2	1	1	1	1	1	5
SRD5A1	1	1	1	1	1	5
TUB	1	1	1	1	1	5
USP45	1	1	1	1	1	5
CBFB	1	1	1	1	1	5
EIF2AK3	1	1	1	1	1	5

METHODS

1. microRNA array and data analysis

For miRNA expression profiling, the small RNA was extracted from tissue samples using the miRVana™ miRNA isolation kit (Ambion). After treated with DNAseI (Qiagen) and checked the quality of RNA by a nano-range bioanalyzer (Agilent Technologies), the miRNA expression profile in tissues was analyzed using the miRCURY LNA Array platform (Exiqon, Woburn, MA, USA) according to the instructions of manufacturers. Briefly, 750 ng RNA of each sample was labeled with Hy3 and Hy5 fluorescent materials (Exiqon, Vedbaek, Denmark), respectively. The Hy3-labeled test sample and Hy5-labeled reference sample were mixed pair-wise and hybridized to the miRCURY LNA array version 14.0 (Exiqon) containing capture probes targeting all miRNAs from all organisms. The hybridization was performed using a Tecan HS4800 hybridization station (Tecan, Austria). The hybridized chips were washed and scanned by using a ScanArray 4000 XL scanner (PackardBiochip Technologies, USA). The ImaGene 6.1.0 software (BioDiscovery, Inc., USA) was used to analyze the image and miRNA expression. A miRNA was considered detected if the spot intensity was at least two standard deviations above background in at least two of the samples. The array data was normalized using the median normalization such that the average intensity in each sample was the same. The gene expression dataset was summarized at Table S4.

2. In situ hybridization (ISH)

The ISH was conducted as previously described (4). Briefly, tissues sectioned to 3–4 μm thickness were pre-treated with nucleic acid retrieval (NAR) solution at 85°C for 5 minutes, 100°C for another 20 minutes, and incubated with protease at 37 °C for 8 min. Then sections were pre-incubated with Exiqon hybridization buffer (Exiqon, Vedbæk, Denmark) at 42°C for 20 minutes and then hybridized with fluorescein labeled miRNA probes for miR-143, miR-145, miR-27b, or miR-193 (100 nM) at 42°C for 2 h. After washed stringently with 5× SSC, 1× SSC and 0.2× SSC buffers at 65 °C for 30 min to remove the non-specific binding probes, the slide was detected by sequential addition of anti-fluorescein antibody followed by poly-HRP. The expression of miRNAs was assessed semi-quantitatively by two of the authors.

The intensity was scored on a scale of 0 - 3 as negative (0), weak (1), medium (2) or strong (3). The extent of the staining, defined as the percentage of positive stained areas of tumor cells per the whole tumor area, was scored on a scale of 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). An overall protein expression score (overall score range, 0 to 12) was calculated by multiplying the intensity and positivity scores. Scores for miRNA staining were dichotomized into two groups; negative (0 to 5) and positive (6 to 12).

3. Cell lines and cell culture

Human lung cancer A549, H1975, H292, H1650, H1299, and human bronchial epithelial cell (HBEC) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in our lab with recommended medium containing 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin (Invitrogen). Human brain microvascular endothelial cells (HBMEC) and pulmonary artery endothelial cells (PAEC) were kindly provided by Prof Tianfeng Chen at Jinan University and cultured in full Dulbecco modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA). Cells were routinely tested for mycoplasma contamination using MycoAlert Mycoplasma Detection Kit.

4. Plasmid construction and generation of stable cell lines

The miR-143-3p lentivirus, anti-miR-143-3p lentivirus and negative control lentivirus (hU6-MCS-Ubiquitin-EGFP-IRES-puromycin, GV309) were purchased from Genechem (Shanghai, China). The human miR-143-3p mimics, miR-143-3p inhibitor and corresponding controls were synthesized by RuiBoBio (Guangzhou, China). The miR-143-3p inhibitor is a single-stranded RNA analogue complementary to the mature miR-143 (5'-3') and modified by cholesterol conjugation from a hydroxyproline-linked cholesterol solid support and 2'-OMe phosphoramidites. Lipofectamine RNAiMAX (Invitrogen) was used for miRNA and its inhibitor transfection.

To generate miR-143-3p stable overexpression cells, A549 cells transfected with the miR-143-3p or negative control lentivirus were selected with 1 μg/ml puromycin for two weeks. The miR-143-3p stable overexpression and control cells were cultured in medium supplemented with 1 μg/ml puromycin. Cells were incubated with medium without puromycin for four days

before experiments. To further generate VASH1 stable over expression cells, control or miR-143-3p stable cells were further transfected with pcDNA 3/.1 or pcDNA/VASH1 via liposome-mediated transfection and selected with G418 (700 μg/ml) for two weeks. The survived cells were picked out and seeded into 96-well plate for formation of cell clones and further expansion. The expanded monoclonal cell populations were named as Control, miR-143-3p o/e, VASH1 o/e, and miR-143-3p/VASH1 o/e cells, respectively.

5. RNA extraction and real-time PCR

RNA extraction with Trizol (Invitrogen) and real time PCR were performed according to the protocol used in our previous study (5). For mRNA quantification, the primers were as follow: GAPDH, forward 5′-GTC TCC TCT GAC TTC AAC AGC G-3’ and reverse 5′-ACC ACC CTG TTG CTG TAG CCA A-3′; VASH1, forward 5′-GGT GGG CTA CCT GTG GAT G-3’ and reverse 5′-CAC TCG GTA TGG GGA TCT TGG-3′; USP54, forward 5′-GAG TTA GAG GCA GCG AAA GGG T-3’ and reverse 5′-TCT TGC AGG GAC CTC TCA AAG C-3′; WASF3, forward 5′- ACC GAT GGC TCC AGC AGA CTA C-3’ and reverse 5′- GCT GAC GAA GGC AGT TTG TGC T-3′; GABRB3, forward 5′- CAG CCA AGG CAA AGA ATG ACC G-3’ and reverse 5′- ATG CCG CCT GAG ACC TCA TTC A-3′; IGFBP5, forward 5′- CGT GCT GTG TAC CTG CCC AAT T-3’ and reverse 5′- ACT TGT CCA CGC ACC AGC AGA T-3′;HK2, forward 5′- GAG TTT GAC CTG GAT GTG GTT GC-3’ and reverse 5′- CCT CCA TGT AGC AGG CAT TGC T-3′; VEGFA, forward 5′- TTG CCT TGC TGC TCT ACC TCC A-3’ and reverse 5′- GAT GGC AGT AGC TGC GCT GAT A-3′; DICER, forward 5′- ACT GCT GGA TGT GGA CCA CAC A-3’ and reverse 5′- GGC TTT CCT CTT CTC AGC ACT G-3′; primary miR-143-3p, forward 5′-GCC AGA GCT GGA GAG GTG GAG-3’ and reverse 5′-AAC TGA CCA GAG ATG CAG CAC TG-3′. GAPDH were used as endogenous control for normalization.

To measure the miRNAs and precursor miR-143-3p, the small RNA was extracted from tissue samples using the miRVana™ miRNA isolation kit (Ambion). qRT-PCR reactions for miRNA detection were quantified by QuantiMir™ RT kit (System Biosciences) according to the instructions. Briefly, all small RNAs were tagged with a poly(A) tail followed by annealing of an oligo(dT) adaptor and cDNA synthesis. Then the miRNAs were quantified by RT-PCRs

using a universal reverse primer and forward primer against specific miRNAs as follows: miR-143-3p, 5′- TGA GAT GAA GCA CTG TAG CTC -3′; miR-145, 5′- GTC CAG TTT TCC CAG GA -3′; miR-27b, 5′- GAG CTT AGC TGA TTG GTG -3′; miR-192, 5′- GAC CTA TGA ATT GAC AGC -3′. The primers for precursor miR-143-3p were: forward 5′- CTG TCT CCC AGC CTG AGG T -3' and reverse 5′- CTC TCT TCC TGA GCT ACA GTG C -3′. 5s rRNA was used as control for normalization with the following primers: forward 5′- TAC GGC CAT ACC ACC CTG AAC-3' and reverse 5′- CGG TCT CCC ATC CAA GTA CTA ACC-3′.

6. Wound healing and *in vitro* invasion assay

The wound healing and in vitro invasion assay were performed according to our previous study (5). For wound healing assay, cells were seeded and cultured until a 90% confluent monolayer was formed. Cells were then scratched by a sterile pipette tip and treated as indicated in text in FBS-free medium. Cell migration distances into the scratched area were measured in 10 randomly chosen fields under microscope. Transwell assay was conducted using CytoSelect™ 24-well Cell Invasion assay kits. Briefly, polycarbonate filters (8 μm pore size, Corning) coated with 50% Matrigel (BD bioscience, Bedford, MA) were used to separate upper and lower chambers. 1×10^5 cells in 200 μl culture medium (supplemented with 0.1% FBS) were added into the upper chamber, while 600 μl medium supplemented with 10% FBS were added into the lower chamber and served as a chemotactic agent. After incubation, cells invading into the lower chamber were fixed, stained and counted under the upright microscope (5 fields per chamber).

7. Western blot analysis

Western blot analysis was performed as previously described (32). The antibodies used in the present study were: MMP2(Bioworld, BS1236, 1:500), FN (Abcam, 2413, 1:500), E-Cad (Bioworld, 3195S, 1:500), Vim (CST, 5741S, 1:1000), VEGFA (Abclonal, A5708, 1:1000), VASH1 (Bioworld, BS9217P, 1:500), deY-Tub (EMD Millipore, AB3201, 1:500), Tubulin (BiOSS, bs-0159R, 1:500), Mettl3 (CST, 96391S, 1:500), Dicer (CST, 5362, 1:500), and GAPDH (BOSTER, BM3876, 1:1000).

8. ***In vitro BBB transmigration assay***

The *in vitro* BBB transmigration assay was performed according to the previous study with slight modifications (6). Briefly, HBMECs (100,000 cells) were plated on 50% Matrigel (BD bioscience, Bedford, MA) coated polycarbonate filters (8 μm pore size, Corning) and cultured for 5 days to allow a confluent monolayer to form. The value of transendothelial electrical resistance (TEER) and cell layer capacitance (C_{CL}) were monitored every hour using a 24-well CellZscope system (7). When the maximal TEER in the range of 20–40 Ωcm^2, A549 cells were added to the top of the insert, and medium containing a chemoattractant was placed in the bottom for the indicated time periods. The transmigrated cells were fixed, stained and counted under the upright microscope (5 fields per chamber).

9. ***Endothelial tube formation assay***

The endothelial tube formation assay was performed according to our previous study (8). Briefly, HUVECs (1×10^5) at passages 2-7 were resuspended in 100 μl of SFM and seeded in 24-well palate coated with Matrigel (R&D Systems, MN, USA). After attachment, HUVEC cells were exposed to condition medium of G-1 treated TNBC cells. Tubes were defined as endothelial cells that had aligned to form >90 % closed structures. Tubes were photographed using inverted phase contrast microscope and quantified by counting the number of connected cells in randomly selected fields under × 10 magnification.

10. Cell proliferation and colony formation

Cell proliferation was tested by CCK-8 kit (Dojindo, Gaithersburg, MD) according to our previous study (9). Colony formation was detected by CytoSelect 96-well Cell Transformation Assay (Cell Biolabs, USA).

11. Luciferase reporter assay

Luciferase assay was performed using reporter lysis buffer (Catalog #E3971, Promega, U.S.A) and luciferase assay reagent according to manufacturer's instructions. Briefly, control or miR-143-3p A549 cells were transfected with pmiRGLO, pmiRGLO-WT-3'UTR, or pmiRGLO-Muta/b/c-3'UTR in a 12 well plate. After transfection for 6 h, each cell line was re-

seeded into a 96 well plate. After 24 h incubation, cells were analyzed with Dual-Glo Luciferase Assay system (Promega). Renilla Luciferase (R-luc) was used to normalize firefly luciferase (F-luc) activity to evaluate reporter translation efficiency.

12. Protein stability

To measure protein stability, cells were treated with cycloheximide (CHX, final concentration 100 μg/ml) during indicated times. The expression of VEGFA was measured through western blot analysis.

13. RNA stability

To measure RNA stability, actinomycin D (Act-D, Catalog #A9415, Sigma, U.S.A) at 5 μg/ml was added to cells. After incubation at the indicated times, cells were collected and RNA was isolated for Real time PCR. Half-life (t1/2) of precursor and mature RNA were calculated using ln2/ slope.

14. Polysome profiling

Polysome profiling was performed according to our previously stated method (10). The fractions were categorized and used to isolate total RNA by Trizol reagent for RT-PCR.

15. Immunoprecipitation

After lysis and centrifuge, input was done with the 2.5 % of the crude lysate. Equal amounts of VEGFA or α-tubulin were immunoprecipitated with a preclearing process and incubated overnight at 4 ºC with the primary antibody and protein A/G as indicated (SCBT, sc-2003). As control, immunoprecipitation with rabbit IgG (SCBT, sc-11390) was also conducted. After 4 washes with lysis buffer and once with PBS plus inhibitors, pellets were resuspended in 6x loading buffer, boiled and loaded onto 8 % polyacrylamide gels and transferred to a PVDF membrane (Immobilon-P, Millipore). The expression of ubiquitin or proteins interacting with α-tubulin was measured by Western blot analysis.

16. Immunofluorescence

Immunofluorescent staining was performed as described previously (9). Briefly, cells growing on the coverslips were washed with PBS, fixed in 4% paraformaldehyde for 15 min and treated with 0.3% triton-x in PBS for 5 min. Cells were blocked with 3% BSA for 1 h at room temperature and incubated with the specific primary antibodies and accordingly dye-conjugated secondary antibody. Finally, cells were counterstained with DAPI (Vector Laboratories, Bulingame, CA). Fluorescent images were acquired using Leica SP8 confocal microscope.

17. Subcellular fraction of RNA

The nucleus and cytoplasm were separated by NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA). The fractions were pooled to isolate total RNA by TRIzol reagent for RT-PCR.

18. m^{6}A-RT-PCR

m^{6}A-RT-PCR was conducted according to previously described protocol with a slight modification (11). Briefly, total RNA was extracted using Trizol. After saving 50 ng of total RNA as input, the remaining RNAs (2 μg) were used for m^{6}A-immunoprecipitation with m^{6}A antibody (Synaptic Systems) in 500 μL IP buffer (150 mM NaCl, 0.1% NP-40, 10 mM Tris, pH 7.4, 100 U RNase inhibitor) to obtain m^{6}A pull down portion (m^{6}A IP portion). m^{6}A RNAs were immunoprecipitated with Dynabeads® Protein A (ThermoFisher Scientific) and eluted twice with elution buffer (5 mM Tris-HCL pH 7.5, 1 mM EDTA pH 8.0, 0.05% SDS, 20 mg/ml Proteinase K). m^{6}A IP RNAs were recovered by ethanol precipitation and RNA concentration was measured with Qubit® RNA HS Assay Kit (ThermoFisher Scientific). Then 2 ng of total RNA and m^{6}A IP RNA were used as template in qRT-PCR as described above. House-keeping gene HPRT1 was chosen as internal control since HPRT1 did not have m^{6}A peaks from m^{6}A profiling data (12).

19. Immunohistochemistry (IHC)

Immunohistochemistry was performed to measure expression of target protein according to our previous study (13).

References for supplementary data

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