Gamma-synuclein promotes Bevacizumab resistance by activating vascular endothelial growth factor receptor in colorectal cancer

Additional file 1

Supplementary Material 1: Supplementary Methods

Preparation of purified SNCG

Purified SNCG from the supernatant of SNCG over-expressing MCF-7-SNCG cells was obtained as previously described (1,2) with some modification. In brief, immobilization of anti-SNCG McAb 1# on Sepharose was prepared by coupling anti-SNCG 1#mAb to cyanogen bromide-activated Sepharose 4B as the manufacturer's recommendations, which was designed for rapid single-step purification of SNCG and was used as the affinity resin to selectively enrich trace amount of SNCG. McAb1#-Sepharose beads were mixed with conditional medium from MCF-7-SNCG and washed with PBS for three times. Bound protein (enriched protein) was eluted from anti-SNCG 1# mAb-Sepharose 4B beads by adding elution Buffer. The eluate was collected and quantified by absorbance at 280 nm or monitored the purity by SDS-PAGE (Figure S1B).

Generation of SNCG-overexpressing tumor cell lines

Expression vectors carrying the Flag-tagged SNCG cDNAs were constructed by sub-cloning the PCR product of human SNCG cDNA into PCDNA3.0 vector and were confirmed by sequencing. pcDNA3-SNCG recombinant plasmid, with flag-tag at the N-end of SNCG, was constructed by Sangon Biotech, Beijing, China. pCDNA3.0-SNCG clone and pCDNA3.0 empty vector was stably transfected into DLD-1, LOVO, and RKO cells using Lipo Transfection Reagent (Promega) and selected with 200-400 μg/ml G418 for two weeks. G418 resistant single clones were isolated and SNCG levels from cell culture supernatant were assessed by a sandwich ELISA and the corresponding clones were successfully established, which were designated as DLD-1-SNCG and DLD-1-C, LOVO-SNCG and LOVO-C, RKO-SNCG and RKO-C,

respectively.

Generation of SNCG-knockout tumor cell lines

To prepare SNCG knockout cells by CRISPR-Cas9 gene editing, sgRNA targeting SNCG gene exon 5 (SNCG (h)-gRNA1, 5′-CAUAGUGGCCGAGAAGACCAagg-3', SNCG (h)-gRNA2, 5′-CACAGCCUCGCUCACGGCGUtgg-3′) was cloned into pGE-2(pU6gRNACas9puro), respectively. For the establishment of stable SNCG knockout cell lines, HT29 and HCT116 cells were cultured for 24 hours to 50%–70% confluence before being transfected with the SNCG-knockout plasmid (ordered from GenePharma, Shanghai, China) using the Lipo Transfection Reagent. Twenty four hours post-transfection, the cells were selected with 2 μg/ml puromycin for two weeks. Efficiency of the knockout was determined by ELISA and Western blot analysis and the successfully established corresponding clones were designated as HT29GKO and HCT116GKO, respectively.

Establishment of cell models resistant to Bevacizumab

To establish the Bevacizumab-acquired resistant cells, human colon cancer cell lines (HT29, HCT116 and DLD-1) were chronically exposed to Bevacizumab for 3 months at a clinically relevant dose (100 μg/ml), based on the US Food and Drug Administration-approved Bevacizumab dose (5 mg/kg) corresponding to a concentration of 100 μg/ml in cell culture experiments. The vehicle-treated control cells were obtained by chronic treatment with PBS for 3 months, and cells adapted to Bevacizumab were named as HT29-Bev, HCT116-Bev and DLD-1-Bev, respectively.

Scratch wound healing assay

HUVEC cells were seeded in 24-well plates at a density of 1 x 10^4 cells per well and

incubated for 24 h. Then a 200 μl pipette tip was used to scratch a linear wound in the cell monolayer. Medium containing SNCG (0.5 μg/ml), VEGF (20 ng/ml), and their combination were added to the corresponding wells. Photographs were taken at 16 h after scrapping and the efficacy of wound healing was assessed using ImageJ (NIH; Bethesda, MD).

Three-dimensional aortic ring assay

Angiogenesis was studied by culturing rings of mouse aorta in three-dimensional collagen gels described as reference (3). Thoracic aortas were removed from mice sacrificed by cervical dislocation and immediately transferred to a culture dish containing ice-cold serum-free RPMI-1640. The peri-aortic fibroadipose tissue was carefully removed with fine microdissecting forceps and surgical clipping paying special attention not to damage the aortic wall. One millimeter long aortic rings were sectioned, extensively rinsed in 5 consecutive washes of RPMI-1640, and embedded in polymerized Matrigel with additional fresh liquid of 60 μl Matrigel. After 3h, 500 μl/well of serum-free medium with or without 0.5 μg/ml SNCG, 20 ng/ml VEGF, and their combination was added. The cultures were kept at 37°C in a humidified environment. After about 6 days of incubation, microvessel-like structures sprouting from the aortic rings were photographed and analyzed using ImageJ.

Chicken embryo chorioallantoic membrane (CAM) assay

CAM assay was performed as previously described (4). Briefly, fresh fertilized SPF white leghorn chicken eggs (Meri Avignon Laboratory Animal Technology Co., Ltd, Beijing, China) were washed mildly with 95% ethanol. After incubation for 3 days in a 60% relative humidified environment at 37℃, eggs were sterilized and opened with the help of an electric drill. The whole egg contents were removed into Petri dishes, and incubated at 37.5℃. Filter papers with the size

of 5.5 mm in diameter were autoclaved and used as carriers for SNCG (0.5 μg/ml), VEGF (20 ng/ml), and their combination were applied to the CAM. At least 5 embryos were used for each group. Then the embryos were cultured for 48 h after drug administration. The vascular response to drugs was estimated using ImageJ\.

Tumor Spheroid Formation Assay

For the preparation of spheroids, cells were harvested to obtain a healthy and single cell suspension. 100 μL of cell suspension with Bev (20 μg/ml), 42# (20 μg/ml) or their combination were dispensed to six wells of Corning™ 96-Well Clear Ultra Low Attachment Microplates (Corning Cat. No. 4520) and incubated at 37 °C and 5 % CO2 for about 48h. The spheroids were examined and photographed via a phase-contrast microscope and the spheroid diameter was measured with imageJ and semiquantified as a histogram.

Surface plasmon resonance (SPR)

SPR analysis was performed by Acrobiosystems Co., Ltd (Beijing, China). Human VEGFR1 (his-tag), Human VEGFR2 (Fc-tag), and Human NRP1 (Fc-tag) were provided by Acrobiosystems Co., Ltd (Beijing, China). SPR is an optical technique used to measure molecular interactions in real time. In an SPR experiment, one molecule (ligand) is immobilized on a sensor chip and binding to a second molecule (analyte) is measured under flow. Response is measured in resonance units (RU) and is proportional to the mass on the surface. Detection methods displayed in supplementary table 1.

References:

1. Liu C, Guo J, Qu L, Bing D, Meng L, Wu J, Shou C. Applications of Novel Monoclonal Antibodies Specific for Synuclein-γ in Evaluating Its Levels in Sera and Cancer Tissues from

Colorectal Cancer Patients. Cancer Lett. 2008;269(1):148-158.

2. Liu C, Shi B, Hao C, Wang Q, Lv Q, Xing N, Shou J, Qu L, Gao Y, Qin C, Zhao J, Shou C. Urine gamma-synuclein as a biomarker for the diagnosis of bladder cancer. Oncotarget. 2016; 7(28):43432-43441. doi: 10.18632/oncotarget.9468.

3. Baker M, Robinson SD, Lechertier T, Barber PR, Tavora B, D'Amico G, Jones DT, Vojnovic B, Hodivala-Dilke K. Use of the mouse aortic ring assay to study angiogenesis. Nat Protoc. 2011;7(1):89-104. doi: 10.1038/nprot.2011.435.

4. Debefve E, Pegaz B, van den Bergh H, Wagnières G, Lange N, Ballini JP. Video monitoring of neovessel occlusion induced by photodynamic therapy with verteporfin (Visudyne), in the CAM model. Angiogenesis. 2008;11(3):235-43. doi: 10.1007/s10456-008-9106-4.

Additional file 2

Supplementary Material 2: Supplementary Table 1

Table S1 Detailed information of antibodies used in Western blot

Antibody	company	Applications	Catalogue No.	Source
Akt	CST	1:5000	#9272	Rabbit, polyclonal
p-AKT(S473)	CST	1:1000	#4060	Rabbit, IgG
ERK	SantaCruz	1:10000	sc-271269	mouse IgG2b
p-ERK1/2	SantaCruz	1:5000	sc-81492	Mouse, IgG1
FAK	CST	1: 2000	#3284	Rabbit, IgG
p-FAK (Y397)	CST	1:500	#8556	Rabbit, IgG
p-FAK (Y925)	CST	1:1000	#3284	Rabbit, polyclonal
GAPDH	Proteintech	1:200000	60004-1-Ig	Mouse, IgG2b
NF-κBp-P65	SantaCruz	1:2000	sc-372	Rabbit, polyclonal
NF-κBp-P65(S536)	SantaCruz	1:1000	sc-33020	Rabbit, polyclonal
p-PLC γ 1	CST	1: 1000	#8713	Rabbit, IgG
p-P38(T180Y182)	CST	1: 1000	#4511	Rabbit, IgG
Src	CST	1: 1000	#2109	Rabbit IgG
p-Src(Y416)	CST	1: 1000	#6943	Rabbit, IgG
SNCG	Our lab	1: 5000	42#	Mouse, IgG1
VEGF	abcam	1: 500	ab46154	Rabbit polyclonal
VEGFR1	GeneTex	1: 500	GTX128969	Rabbit, IgG
p-VEGFR1(Y1213)	GeneTex	1: 1000	GTX32184	Rabbit, IgG
VEGFR2	CST	1: 1000	#2479	Rabbit, IgG
p-VEGFR2(Y1175)	CST	1: 1000	#2478	Rabbit IgG
p-VEGFR2(Y1175)	GeneTex	1: 1500	GTX32403	Rabbit polyclonal
p-VEGFR2(Y951)	GeneTex	1: 2000	GTX50153	Rabbit polyclonal

Additional file 3

Supplementary Material 3: Supplementary Table 2

Table S2 Experimental conditions used in immunohistochemistry

Abtibody	Manufacture	Dilution	Antigen Retrival
CD31(Rabbit IgG, #77699)	CST	1:100	Sodium citrate buffer (pH,6.0), WB at 95°C for 20minutes
VEGFR1 (Rabbit IgG, GTX128969)	GeneTex	1:1200	EDTA, pH9.0, microwave 95℃ for 20min
p-VEGFR1(Y^{1213}) (Rabbit IgG, GTX32184)	GeneTex	1:2000	EDTA, pH9.0, microwave 95℃ for 20min
VEGFR2 (Rabbit IgG, #2479)	CST	1:500	EDTA, pH9.0, microwave 95℃ for 20min
p-VEGFR2(Y^{1175}) (Rabbit polyclonal, GTX32403)	GeneTex	1:20000	EDTA, pH9.0, microwave 95℃ for 20min
p-VEGFR2(Y^{951}) (Rabbit polyclonal, GTX50153)	GeneTex	1:1000	EDTA, pH9.0, microwave 95℃ for 20min
VEGF (Rabbit polyclonal, ab46154)	abcam	1:1000	EDTA, pH9.0, microwave 95℃ for 20min
SNCG (mouse IgG1, 42#)	Our lab	1:2000	EDTA, pH9.0, microwave 95℃ for 20min

Additional file 4

Supplementary Material 4: Supplementary Table 3

Table S3 Target siRNA sequences against VEGFR2, and VEGF

Gene	sense（5'-3'）	antisense（5'-3'）
VEGFR2-homo-1137	GGCAUGUACUGACGAUUAUTT	AUAAUCGUCAGUACAUGCCTT
VEGFR2-homo-2941	GUCCCUCAGUGAUGUAGAATT	UUCUACAUCACUGAGGGACTT
VEGFR2-homo-3089	GCACGAAAUAUCCUCUUAUTT	AUAAGAGGAUAUUUCGUGCTT
VEGFA-homo-1184	GCAGCUACUGCCAUCCAAUTT	AUUGGAUGGCAGUAGCUGCTT
VEGFA-homo-1359	GCGGAUCAAACCUCACCAATT	UUGGUGAGGUUUGAUCCGCTT
VEGFA-homo-1408	CAGCACAACAAAUGUGAAUTT	AUUCACAUUUGUUGUGCUGTT
GAPDH Positive control	UGACCUCAACUACAUGGUUTT	AACCAUGUAGUUGAGGUCATT

Additional file 5

Supplementary Material 5: Supplementary Figure 1

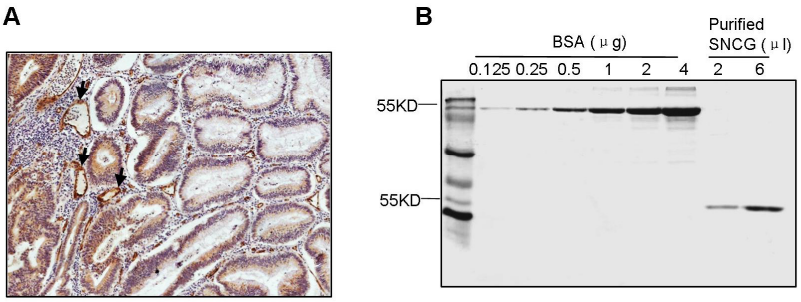

Figure S1. SNCG is over-expressed in tumor vasculature and cancer cells in CRC

A, Representative immunohistochemical staining for SNCG (brown) with haematoxylin counterstain in human colon adenocarcinoma tissues. Arrows, blood vessels with positive SNCG staining. **B**, Identification of purified SNCG on SDS-PAGE.

Additional file 6

Supplementary Material 6: Supplementary Figure 2

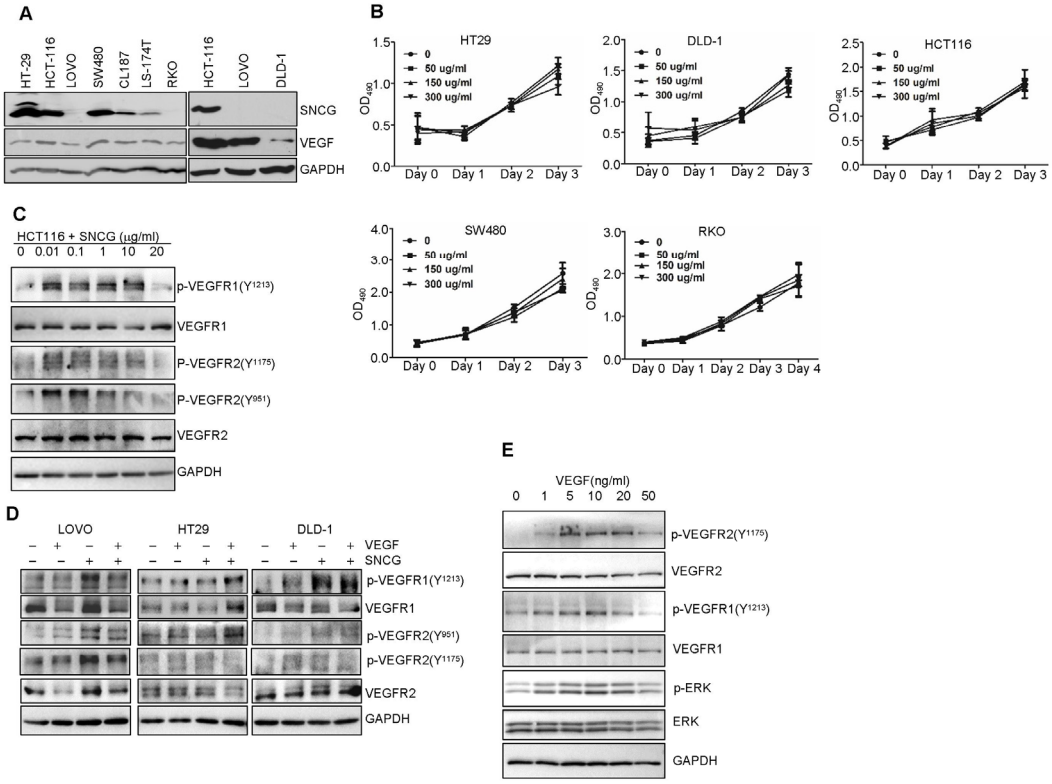

Figure S2. SNCG up-regulates p-VEGFR in CRC cell lines

A, Levels of SNCG，VEGF(R) were detected in indicated CRC cell lines. **B**, The effect of Bev on growth of CRC cell lines as indicated. Cells were treated with various concentrations of Bev, and cell densities were detected at different time point. **C**, HCT116 cells were serum starved overnight and treated with SNCG with various concentrations for 3h and p-VEGFR levels were detected by Western blot. **D**, CRC cell lines were serum starved overnight and treated with 20 ng/ml SNCG or 20 ng/ml VEGF for 3h and p-VEGFR levels were detected by Western blot. **E**, HUVEC was serum starved overnight and treated with VEGF with various concentrations as indicated for 10 min and p-VEGFR levels were detected by Western blot.

Additional file 7

Supplementary Material 7: Supplementary Figure 3

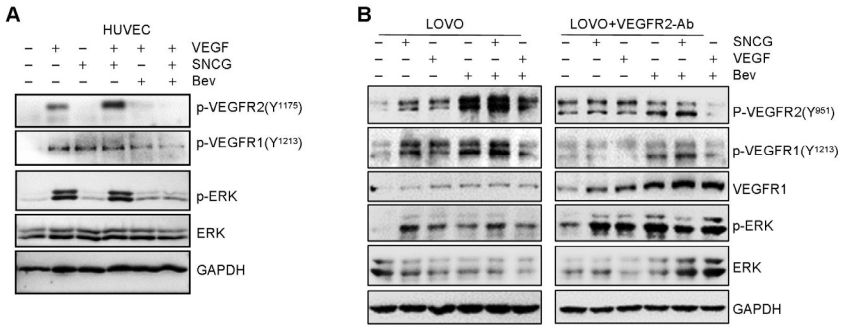

Figure S3. Role of SNCG in the effect of Bev on the level of phosphorylated VEGFR2 in HUVEC or CRC cell lines

A, HUVEC cells were starved overnight and treated with VEGF (20 ng/ml), SNCG (0.5 μg/ml), Bev (500 ng/ml), and the combinations as indicated for 20 min. Cell lysates were collected and detected by Western blot. **B**, LOVO cells were treated with or without neutralizing anti-VEGFR2 antibody for 2h, then cells were treated with 10 ng/ml SNCG, 10 ng/ml VEGF, or 1 μg/ml Bev or the combination as indicated for 3h. Cell lysates were collected and detected by Western blot.

Additional file 8

Supplementary Material 8: Supplementary Figure 4

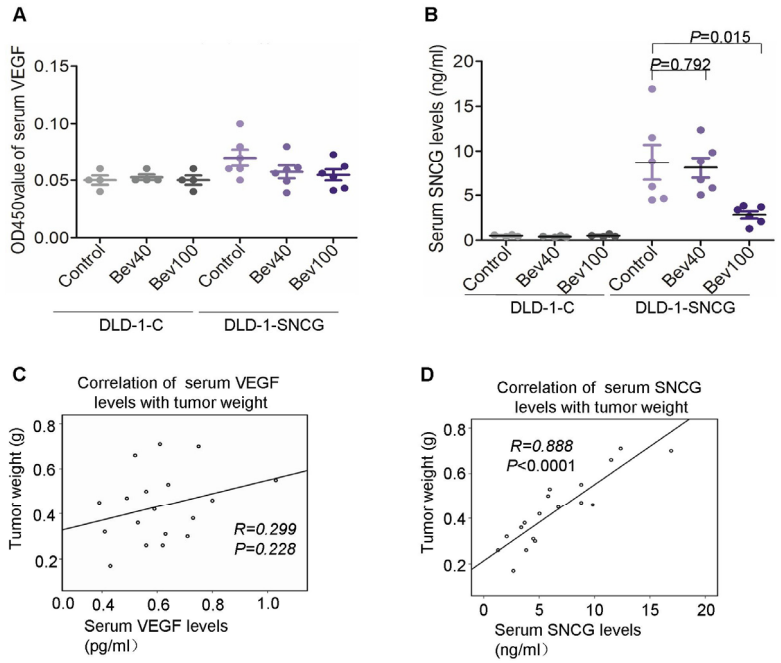

Figure S4. Correlation between serum SNCG, VEGF levels and tumor size in DLD-1/DLD-1-SNCG tumor-bearing mice treated with Bev

A-B, Scatter plot of serum VEGF levels (A) or SNCG levels (B) in mice bearing subcutaneous tumors from DLD-1 and DLD-1-SNCG cell lines treated with Bev. **C-D**, Correlation between serum VEGF (C), SNCG (D) levels with tumor weight of DLD-1 and DLD-1-SNCG xenografts treated with Bev.

Additional file 9

Supplementary Material 9: Supplementary Figure 5

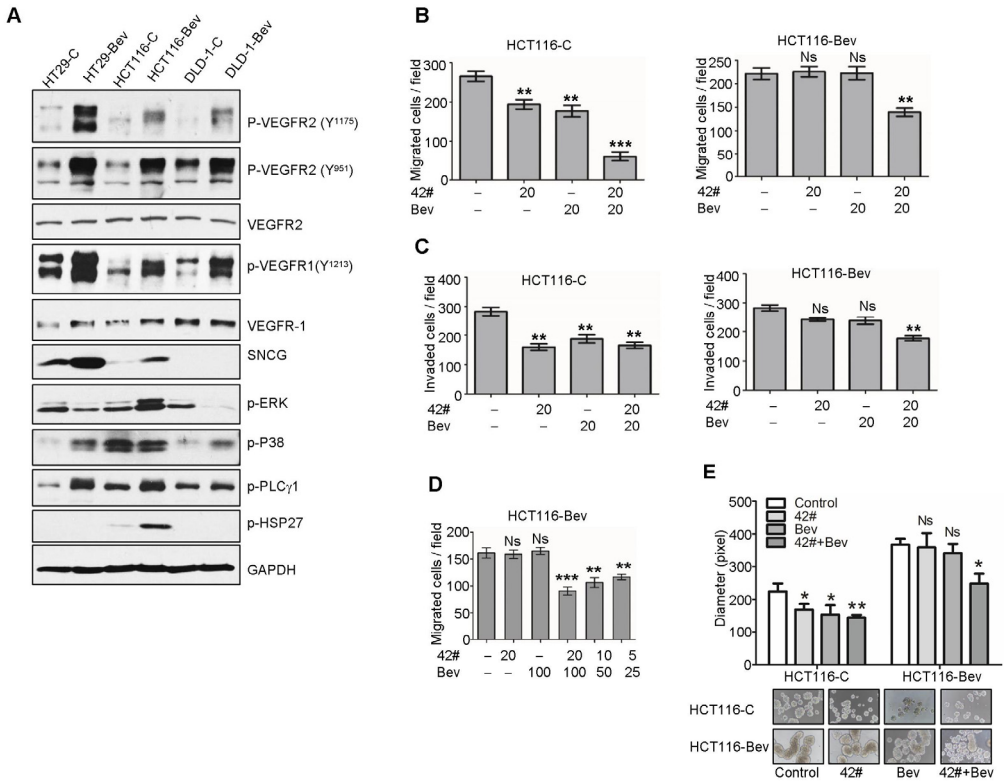

Figure S5. Combining 42# with Bev reverses Bev-acquired resistance in CRC cells

A, Levels of phosphorylated VEGFR and the downstream proteins were detected by Western blot for the Bev-acquired resistant cell lines HT29-Bev, HCT116-Bev, DLD-1-Bev and their corresponding parental cells. **B-C**, Migration (B), invasion (C) of HCT116-C (left panel) and HCT116-Bev (right panel) cells treated with 42#, Bev, or their combination. **D**, Migration of HCT116-Bev treated with 42#, Bev, or various concentrations of combined 42# with Bev. **E**, Spheroid formation of HCT116-C and HCT116-Bev treated with 20 μg/ml 42#, 20 μg/ml Bev, or their combination. Spheroid diameter was measured with imageJ and semi-quantified as a histogram (top panel). Representative images of spheroid formation were showed (bottom panel). Ns, no significant difference; *$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$.

Additional file 10

Supplementary Material 10: Supplementary Figure 6

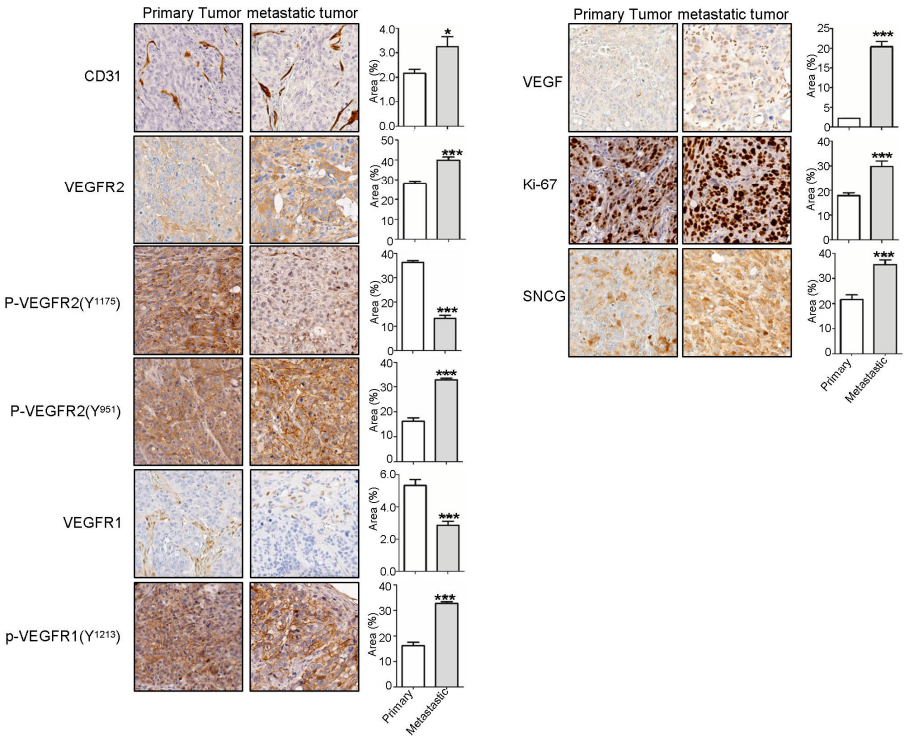

Figure S6. **Difference of protein expression for primary and metastatic HT29 xenografts by immunohistochemistry**

Sections from primary and metastatic HT29 xenografts were immunohistochemically stained with CD31, Ki-67, VEGFR1, p-VEGFR1(Y^{1213}), VEGFR2, VEGFR2(Y^{1175}), VEGFR2(Y^{951}), VEGF, and SNCG. Six random microscopic fields per tumor were taken and the amount of positive staining was calculated using ImageJ. Representative images (200x) (left panel) were shown and intensity of staining (right panel) was reported as a relative positive area. Ns, no significant difference; *$P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

Additional file 11

Supplementary Material 11: Supplementary Figure 7

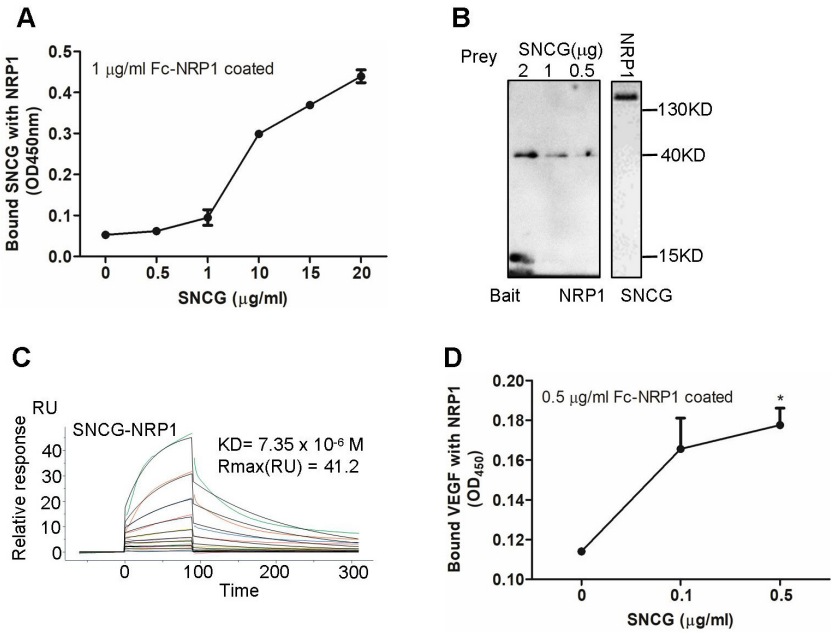

Figure S7. SNCG binds to VEGF coreceptor neuropilin-1 (NRP1).

A-C, Interaction of SNCG with NRP1 was detected by ELISA (A), Far-western blot (B), and SPR (C). NRP1 (hFc-tag) captured on Protein G Chip associated with SNCG with an affinity constant of 7.35 μM as determined in SPR assay. **D**, SNCG enhanced binding of VEGF with NRP1. 0.5 μg/ml NRP1 was coated on ELISA plate, the binding ability of VEGF was detected in the presence or absence of SNCG in various concentrations.

Additional file 12

Supplementary Material 12: Supplementary Figure 8

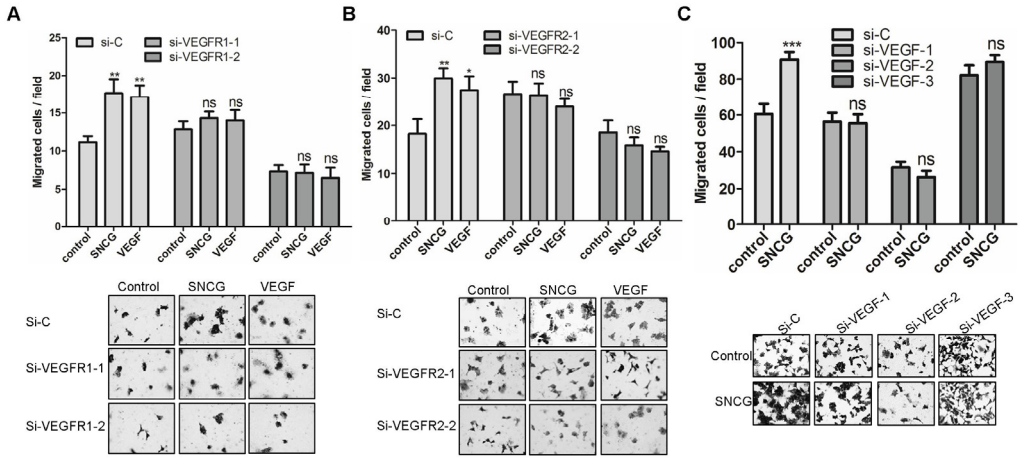

Figure S8. VEGF, VEGFR1, and VEGFR2 are required in SNCG promoting tumor cell migration

A-C, VEGFR-1 (A), VEGFR-2 (B) or VEGF (C) were knocked down by siRNA in LOVO cell line, then cells were subjected to migration assay in the presence of SNCG or VEGF. The histogram (top panel) of migrated cells and representative images of migration (bottom panel) were shown. Ns, no significant difference; **P* < 0.05; ***P* < 0.001; ****P* < 0.0001.