Supplementary Table 1 Antibodies used in this study

Antibody	Company	Country	Clone Number	Species	Dilutions
HIF-1 α	abcam	Cambridge, UK	H1alpha67	Mouse	1:50
VEGF	Santa cruz	California, USA	C-1	Mouse	1:100
VEGFR1	Labvision	Michigan, USA	polyclonal	Rabbit	1:100
MMP-2	Labvision	Michigan, USA	polyclonal	Rabbit	1:50
MMP-9	abcam	Cambridge, UK	polyclonal	Rabbit	1:200
VE-Cadherin	abcam	Cambridge, UK	polyclonal	Rabbit	1:500
Endomucin	E-bioscience	California, USA	eBioV.7C7	Rat	1:400
Caspase3	abcam	Cambridge, UK	polyclonal	Rabbit	1:500
CD68	Zymed	California, USA	KP1	Mouse	Ready to use
CD31	Zymed	California, USA	JC70A	Mouse	Ready to use
β -actin	Santa cruz	California, USA	polyclonal	Rabbit	1:200

Supplementary Methods

Animals

The study has been approved by the Experimental Animal Care and Use Committees. Six-week-old male C57/BL mice (18～22g) were purchased from the Animal Center Academy of Military Medical Science (Beijing, China) and were bred under specific pathogen-free conditions. The tumor bearing animal model was built by injection of a suspension of B16 melanoma cells (4×10^5/0.1 ml) into the hind limb or groin areas of the mice.

20 mice were randomly divided into the following two groups with 10 mice per group. A suspension of B16 melanoma cells was injected into the left skeletal muscle of one group while the other was injected into the abdominal cavity. Mice were sacrificed 9 days after tumor inoculation.

Another 30 mice were used to be inoculated with B16 melanoma cells into the left groin area subcutaneously. 10 mice were sacrificed respectively after 14, 19 and 24 days of tumor inoculation.

Besides, another 60 subcutaneous B16 melanoma bearing mice were divided into the following 6 groups with 5 mice per group for tumor volume monitoring and another 5 mice per group for tissue collection: (1)solvent control (DMSO); (2) B16 cells transfected with siRNA control; (3)AC-DEVD-CHO; (4) ZM-IETD-FMK; (5)ZM-LEHD-FMK; (6)B16 cells transfected with siRNA Fas. The caspase 3 inhibitor, (AC-DEVD-CHO), caspase 8 inhibitor (ZM-IETD-FMK), and caspase 9 inhibitor (ZM-LEHD-FMK) were from Sigma–Aldrich (Missouri, USA). All above mentioned inhibitors were reconstituted in dimethyl sulfoxide(DMSO). Synthetic oligonucleotide sequences for generation of shRNA targeting Fas (5-GTG CAAGTG CAA ACC AGA C-3) and non-specific control (scrambled) were synthesized (FulenGen Company, Guangzhou, China）and ligated into psiHIV-H1 vector to create psiHIV-H1-Fas, psiHIV-H1-control. The vectors were transfected into B16F10 cells following the manufacturer's protocol (Lenti-Pac™ HIV Expression Packaging Kit) and more than 95% of control or siRNA transfected cells showed strong green fluorescence by microscopy. The inhibitors mentioned above were administered intraperitoneally daily for 7 consecutive days from the eighth day of tumor inoculation according to the body weight (1mg/kg). Tumor volumes (V) were monitored and calculated from the formula, $V = \text{length} \times \text{width}^2 \times 0.5$. Tumor tissues were collected and excised for paraffin processing before been sectioned into slices of

5μm thick for histological studies by hematoxylin and eosin (H & E) and immunohistochemistry (IHC) staining.

For the detection of hypoxic areas in the tumors transplanted in the groin areas of mice(n=10, built as mentioned above), the hypoxia marker pimonidanole (Hypoxyprobe-1 Kit, HPI, USA) was used by intraperitoneal injection at a dosage of 60mg/kg body weight 90 minutes before sacrificing the mice on the 14^{th} day of tumor inoculation. The slides of the tumor tissue were processed as the IHC methods below except that an anti-pimonidanole mouse IgG1 monoclonal antibody in the kit was used as the primary antibody.

Tumor bearing hind limb ischemia model

The hind-limb tumor bearing animal model was established as mentioned above by injection of suspension of B16 melanoma cells into the right limb after which the right hind limb was made to be ischemia by the resection of the femoral artery either below the bifurcation of the deep femoral artery (A-group, n=10) or from the distal site of the bifurcation of the deep femoral artery to the saphenous artery (A-strip group, n=10). Non ischemic treated implanted tumors were as control group (n=10).This hind-limb ischemia model was posed by Goto(18) in which the A-group was proven to be a chronic mild ischemia model while the A-strip group was a stable severe ischemia model.

Primers used in this study

The forward primer for Bax was 5′-GGTTTCATCCAGGATCGAGACGG-3′, the reverse primer for it was 5′ -ACAAAGATGGTCACGGTCTGCC-3′ .

The forward primer for Bcl-2 was 5′-ACAGGGGCTACGAGTGGGAT-3′, the reverse

primer for it was 5′-CTCAGTCATCCACAGGGCGA-3′.

The forward primer for Caspase-9 was 5′-ATGGACGAAGCGGATCGGCGGC-3′and the reverse primer for it was 5′-TTA TGA TGT TTT AAA GAA AAG-3′.

The forward primer for Caspase-8 was 5′-GGCATCTGCTTTCCCTTGTTC-3′ and the reverse primer for it was 5′-ATCTTACGACGACTGCACTGC-3′

The forward primer for Fas was 5′-GAGAATTGCTGAAGACATGACAATCC-3′ and the reverse primer for it was 5′-ATGGCTGGAACTGAGGTAGTTTTCAC-3′.

The forward primer for Calpain1 was 5'-CGGTTGGAGGAGGTGGATGA and the reverse primer for it was 5'-ATCACGCTTCAAGTGCACAG-3'.

The forward primer for Cytochrome c was 5'-ACGTGTCGACCTAATATGGGTGATGTTGAAAAAGG and antisense primer was 5'-ACAGATCTTTCTCATTAGTAGCCTTTTTAAG-3'.

The forward primer for VE-Cadherin was 5′-CCGGCGCCAAAAGAGAGA-3′，and the reverse primer for it was 5′-CTGGTTTTCCTTCAGCTGGAAGTGGT-3′.

The forward primer for HIF-1α was 5'-AGCCCTAGATGGCTTTGTGA-3', and the reverse primer for it was 5'-TATCGAGGCTGTGTCGACTG-3'.

The forward primer for MMP-2 was 5'-CCCCTATCTACACCTACACCAAGAAC-3' and the reverse primer for it was 5'-CATTCCAGGAGTCTGCGATGAGC-3'.

The forward primer for VEGF was 5'-CAGGCTGCTCTAACGATGAA-3' and the reverse primer for it was 5'-CAGGAATCCCAGAAACAACC-3'.

The forward primer for VEGFR1 was 5′-CGGAAGGAAGACAGCTCATC-3′, and antisense primer was 5′-CTTCACGCGACAGGTGTAGA-3′.

Glyceraldehyde 3-phosphate dehydrogenase was used to normalizethese (forward primer, 5 ' -CCTGGCCAAGGTCATCCATGAC-3 ' ; reverse primer, 5 ' -TGTCATACCAGGAAATGAGCTTG-3 ').

Methods of vector quantity distribution of LPPCN and blood vessels

The representative tumor regions were selected and marked on the donor blocks. The selected areas were punched into a cylinder by a 1 mm diameter hollow needle, and were inserted into an empty paraffin block. Subsequently, these blocks were cut into 100 serial slides (4 μm thickness), and were prepared for CD31 staining. The

immunohistochemistry slide images were recorded using an Olympus BX51 microscope. The microvessels appeared as brown stains in the images, whereas the tumor cells were negatively stained with CD31. The nucleus heterogeneity of LPPCN cells and their affinity to hematoxylin distinguish them from the tumor cells around them. The grayscale threshold value of the tumor tissue was adjusted. The pixel values of LPPCN cells and blood vessels were identified based on the different slides. For each pixel point in the image, the force applied by all other pixels in the image was calculated and summed into a resultant direction vector. About 100 serial sections of the 30 melanoma tissue microarray blocks were divided into sections of 42 small units, such that 30 matrices were obtained. Each matrix was composed of 100 rows and 42 columns. The mean values of the vector matrices of the microvessels and LPPCN cells are listed in the Supplementary Data. Vector mean value curves for the microvessels and LPPCN cells were constructed from the data. Cell pixel was treated as a low gray value images while vascular image is processed for high gray value images. The medical three-dimensional graphics modeling system (3D-DOCTOR 4.0 version) was used for overlay and volume rendering of the tissue images after accurate registration of 100 continuous slides. The pseudo-color was adjusted to distinguish different regions in the images.

Caspase inhibitors and shRNA construction

The caspase 3 inhibitor, (AC-DEVD-CHO), caspase 8 inhibitor (ZM-IETD-FMK), and caspase 9 inhibitor (ZM-LEHD-FMK) were from Sigma–Aldrich (Missouri, USA). All above mentioned inhibitors were reconstituted in dimethyl sulfoxide(DMSO). Synthetic oligonucleotide sequences for generation of shRNA targeting Fas (5-GTG CAAGTG CAA ACC AGA C-3) and non-specific control (scrambled) were synthesized (FulenGen Company, Guangzhou, China）and ligated into psiHIV-H1 vector to create psiHIV-H1-Fas, psiHIV-H1-control. The vectors were transfected into B16F10 cells following the manufacturer’s protocol (Lenti-Pac™ HIV Expression Packaging Kit) and more than 95% of control or siRNA transfected cells showed strong green fluorescence by microscopy.

Immunohistochemistry methods

The excised sections were pretreated with citrate buffer (0.01M citric acid; pH 6.0) for 10 min at 95°C in a microwave oven. Nonspecific binding sites were blocked by exposure to 10% normal goat serum for 20 min at room temperature. The sections were incubated overnight at 4°C with a series of primary antibodies (Supplementary Data Table 1). The staining systems used were PicTure PV6001 and PV6002 (Zhongshan Chemical Co., Beijing, China). The slides were then incubated with 3, 3′-diaminobenzidine (DAB) chromogen for 5–10 min and counterstained with hematoxylin. PBS was used in place of the primary antibodies as the negative control.

Laser capture microdissection (LCM)

8μm frozen tissue sections were cut onto ethanol-dipped glass slides and placed on dry ice until subsequent fixation (70% ethanol for 1 minute) , H&E staining (Mayer's hematoxylin for 30 seconds, ddH_2O for 10 seconds, eosin for 10 seconds) and dehydration (70% ethanol for 30 seconds, 100% ethanol for 1 minute, xylene for 2 × 5 minutes). Target cells were selected based on the morphology of tumor cells. The LPPCN cells whose nucleus and cell volume were condensed and linearly arrayed in the slides and their neighboring cells as well as the nomal tumor cells were selected in the 100 magnification field before microdissection was performed. The total RNA (from the tissue collected by LCM) was isolated using TRIzol reagent (Invitrogen Life Technologies). Reverse transcription (TaKaRa Biotechnology Co., Ltd., Japan) and PCR(polymerase chain reaction analysis, 2×Taq PCR MasterMix, Beijing,China) were used to evaluate the mRNA levels of Bax, Bcl-2, Cytochrome c, Caspase3, Caspase9, Calpain1, Caspase8, Fas, HIF-1α ,VEGF, VEGFR1, VE-Cadherin and GAPDH. Primer and probe sets were designed and synthesized (Supplementary Data). β-actin mRNA was used as an endogenous control to correct for potential variations in RNA loading or amplification efficiency.

Cell viability

B16F10 melanoma cells were seeded on a 96-well plate (3×10^4 per well) in 100 μl RPMI 1640 complete medium containing 0.2mmol/l DEVD or LEHD in a 96-well plate. After incubation for the desired time, 4 ml of MTT [3-(4,5-dimethylthiazol-2,5-diphenyl) tetrazolium bromide] reagent [5mg/ml in phosphate-buffered saline (PBS)]

was added to each well and incubated for 4h, 37℃. The MTT solution was removed, the formazan crystals were dissolved in 100 μl of DMSO and the absorbance was recorded on a microplate reader at 490 nm.

Wound healing and Transwell assays

B16F10 melanoma cells (1×10^5) in 100 μl RPMI 1640 medium without FBS were seeded onto Matrigel (5mg/ml, BD Bioscience, California, USA) coated upper wells with 8 μm porosity polyethylene terephthalate filters (Invitrogen, California, USA) after trypsinization. The lower inserts used 10% FBS media. After 24 h of incubation at 37 °C, the upper surface of the membrane was wiped to remove non-migratory cells. The cells that invaded through the Matrigel and adhered to the membrane bottom were stained with crystal violet solution. The cell-associated dye was eluted with 10% acetic acid, and its absorbance at 570 nm was recorded. Wound healing assays were performed to assess the movement of cells into a scraped wound. The speed of wound closure was monitored after 18 and 36 h by measuring the ratio of the size of the wound at 0 h. Each experiment was also performed in triplicate.

Three-dimensional cultures

96-well plates were coated with 50 μl Matrigel which was allowed to gel (1 h, 37°C). B16F10 cells were seeded in complete RPMI1640 medium onto the gel and incubated at 37°C for 24 h. Capillary-like structure formation was filmed under phase contrast microscope (40×).

Zymography Assays

Culture media were collected and sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 0.01% wt/vol gelatin containing 10% polyacrylamide gel was used. Following electrophoresis, the SDS was removed from the gel by incubation in 2.5% Triton X-100, followed by incubation in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 150 mM NaCl, 1 mM $ZnCl_2$, and 0.02% NaN_3 for 42 hours at 37°C. The gels were subsequently stained with Coomassie R250, and areas of digestion appear as clear bands against a darkly stained background where the substrate has been degraded by the enzyme.

Western-blotting analysis

The whole cell lysates were resolved by way of sodium dodecyl sulfate – polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore). Blots were blocked and incubated with the monoclonal antibody (Supporting Table 1), followed by incubation with a secondary antibody (1:2,000; Santa Cruz Biotechnology).Blots were developed using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway,NJ). For protein loading analyses, a monoclonal beta-actin antibody (1:200; Santa Cruz Biotechnology) was used.

Statistical analysis

One-Way ANONA analysis of variance was performed to compare the equality of three or more means while a paired sample t-test was used to determine the differences between the average values of the same measurement made under two different conditions. Wilcoxon rank-sum was used when the population cannot be assumed to be normally distributed.

Image acquisition tools and processing software

Syngene imaging systems was used to analyze the electrophoresis gel. Nikon ECLIPSE 90i/Ti microscope and imaging software NIS-Elements version 3.00 were used to acquire microscopic images. Coredraw 9.0 was used to puzzle the images.

Supplementary results

Mean values of the vector matrices of LPPCN and microvessels

1. mean values of the vector matrices of the microvessels：1rows and 42 columns

5.4169e-005	0.00041962	0.00071106	3.7384e-005	0	0
0	0.00020752	5.3406e-005	0.0012581	0.00030212	0.00026398
0	0.00033646	0.00036011	0.00021591	8.8501e-005	0.00033417
4.5776e-006	9.7656e-005	9.9182e-006	0.00031281	0.00081177	6.179e-005
7.6294e-006	0.00028687	0	0.00034027	0.001667	0.00097656
0.00055237	0.0013863	0.00025024	0.00080032	0.00043411	0
0.00014267	6.4087e-005	0	0	1.1444e-005	7.401e-005

2. mean values of the vector matrices of the LPPCN cells：1rows and 42 columns

0.00067215	0.0019287	0.00052261	0.00024414	4.9591e-005	0
0	0.0012177	0.0010056	0.0010803	0.00063858	0.0005043
3.9673e-005	0.00012665	0.0014725	0.00087509	0.00065918	0.00043335
6.0272e-005	0.00042267	0.00010986	0.0016304	0.0009613	0.00034943
0.00027771	0.00072098	0.00014954	0.0010483	0.0013542	0.0014893
0.00090942	0.0012535	0.00045776	0.00093155	0.0010674	4.8828e-005
0.00052795	0.0002121	4.7302e-005	9.9182e-006	0.00013809	3.8147e-005

Supplementary Figure legends

Supplementary Figure 1. The LPPCN cells were captured by LCM. The representative images were taken and the original magnification is ×40.