Supplementary information

Targeting *TRMT5* suppresses hepatocellular carcinoma progression via inhibiting the HIF-1α pathways

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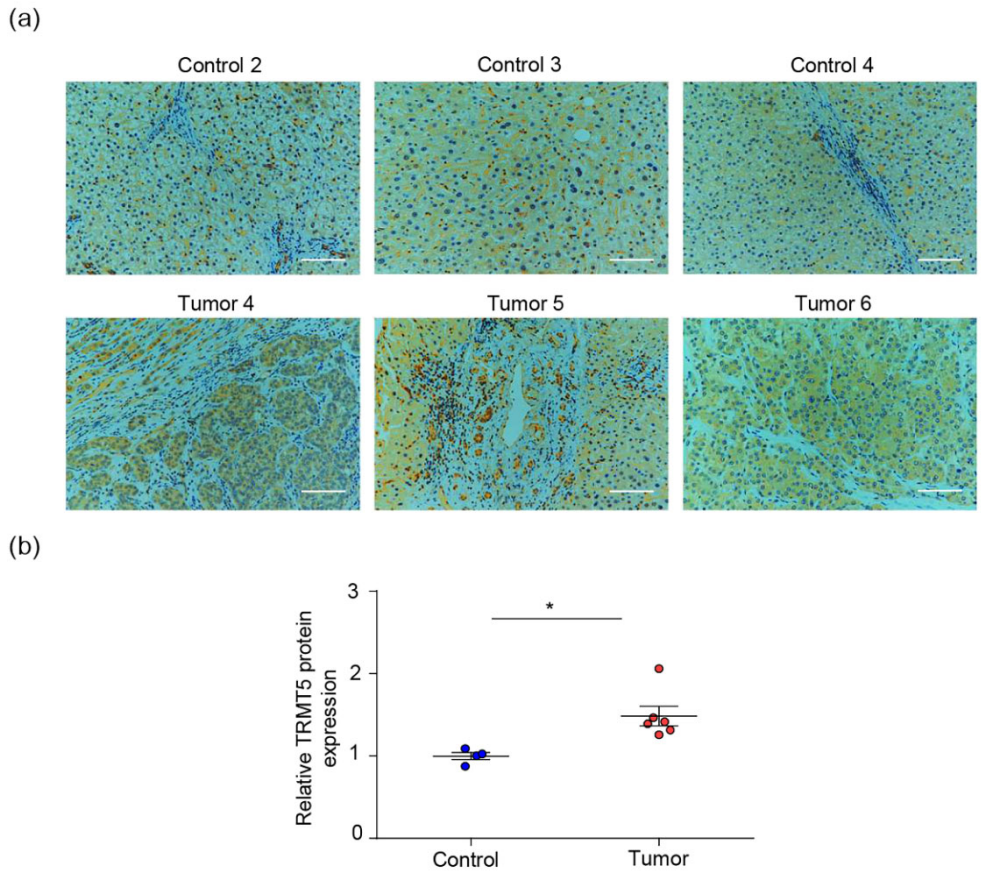

Fig. S1 The expression level of TRMT5 in patient samples. (a) Immunohistochemical staining for TRMT5 protein was performed in 3 normal liver tissue and 3 tumor tissues from HCC patients. (b) Quantification of relative TRMT5 expression. *P* indicates the significance (P*<0.05).**

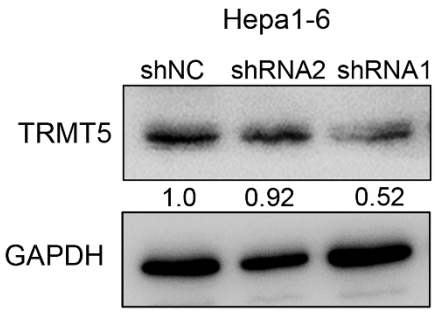

Fig. S2 The expression levels of TRMT5 in shRNA1, shRNA2, and shNC Hepa1-6 cells. The expression level of shNC cell was considered as 1, and the values for the silenced cells were expressed as ratio of the control cell value.

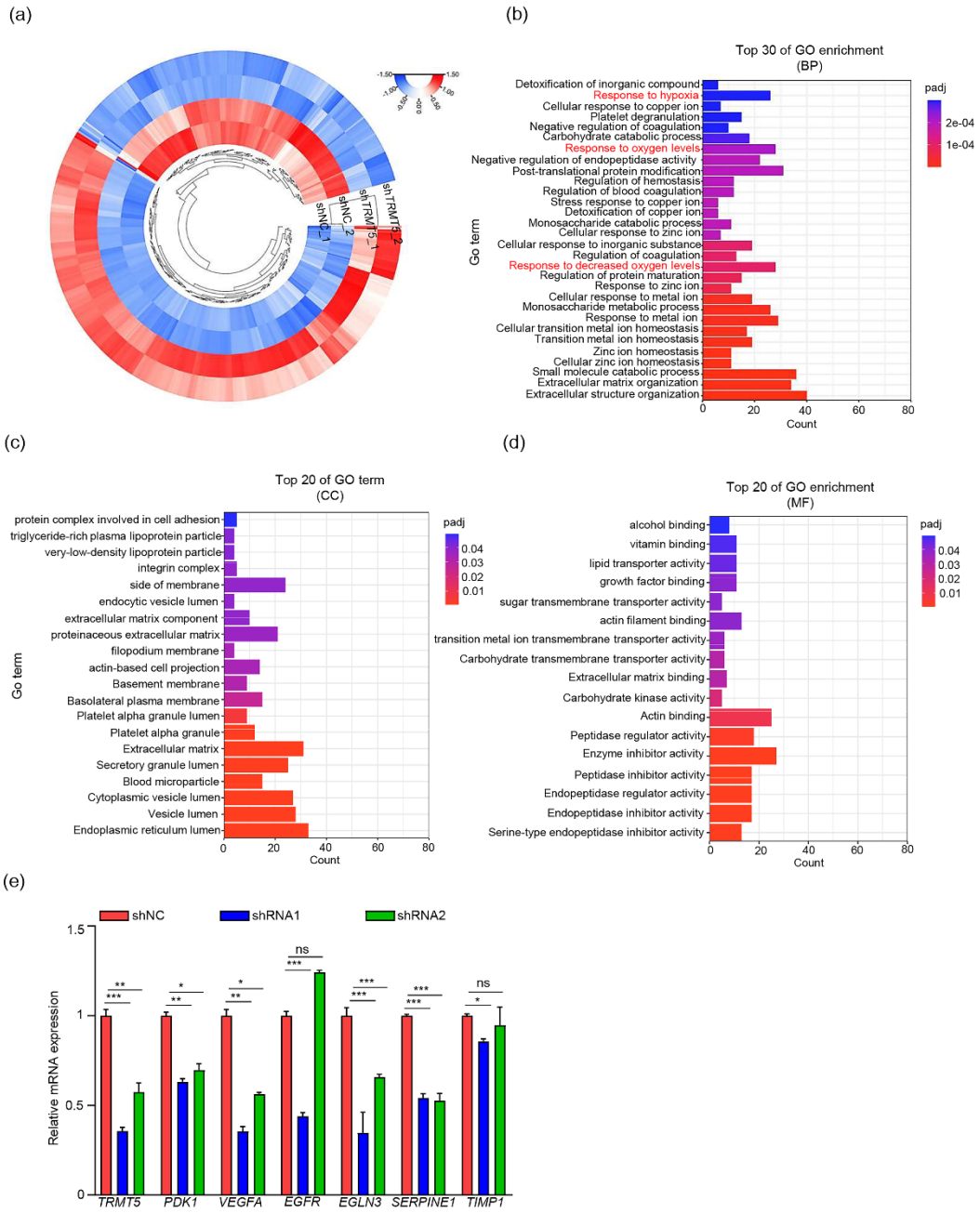

Fig. S3 RNA sequencing analysis between shRNA1 and shNC HepG2 cells. (a) Heat map of differentially expressed genes. (b) Top 30 GO terms of biological process. BP, biological process. (c) Top 20 GO terms of cellular component (CC). (d) Top 20 GO terms of molecular function (MF). (e) qRT-PCR analysis detected the mRNA expression level of representative dysregulated genes in the HIF-1 signaling pathway. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, and ns, no significant difference.

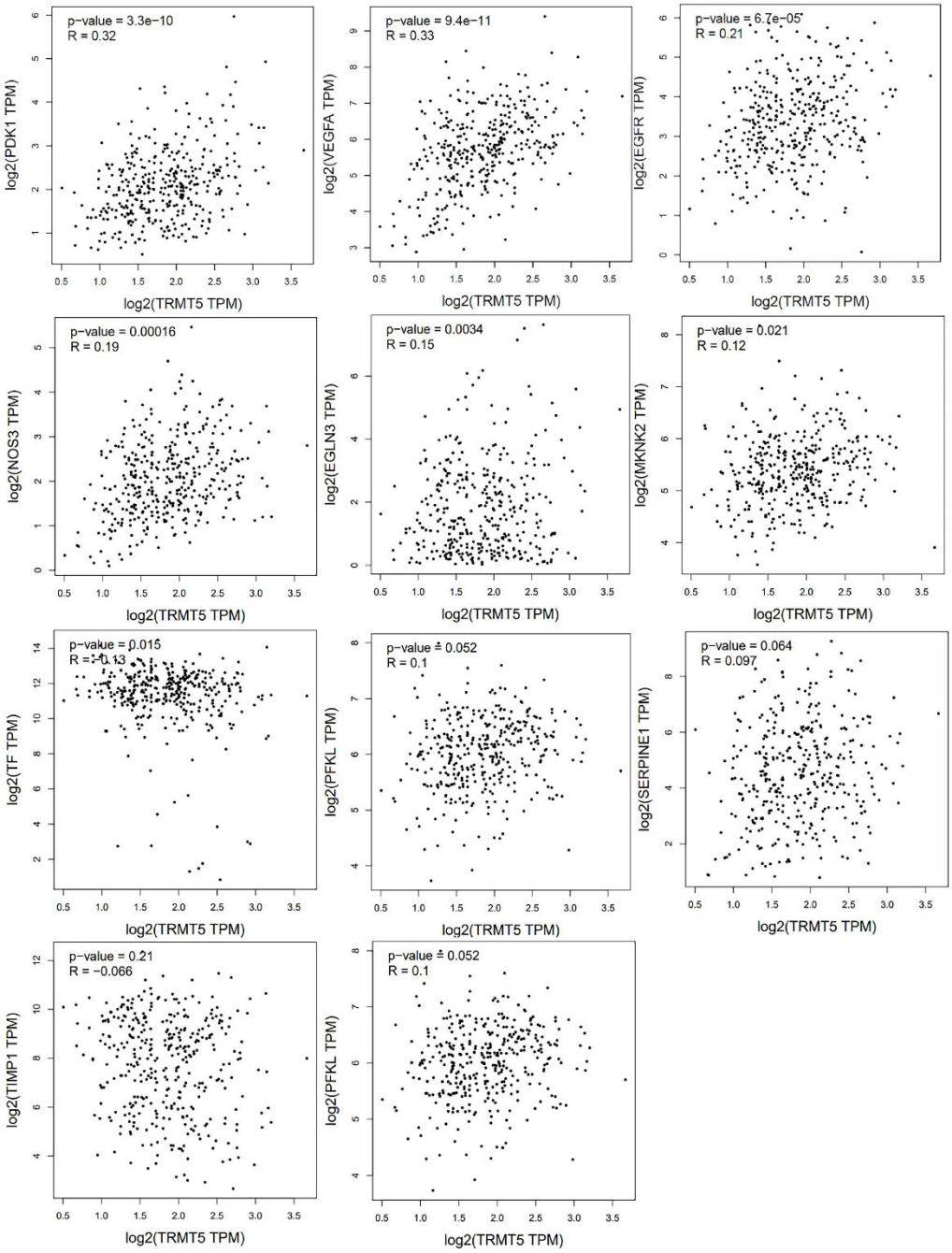

Fig. S4 The correlation of the mRNAs of TRMT5 and genes in HIF1 signaling pathway.

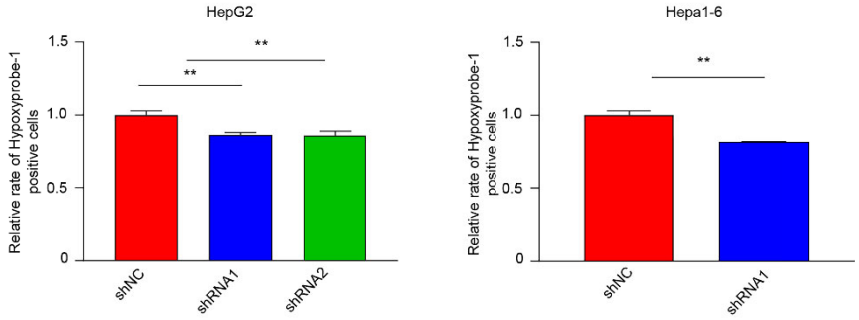

Fig. S5 The quantification of hypoxyprobe-1 positive cells in HepG2 and Hepa1-6.

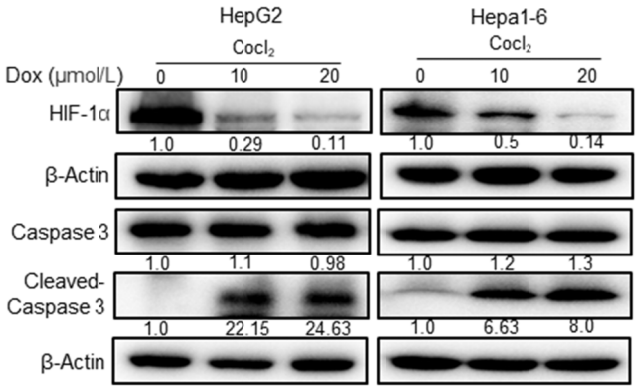

Fig. S6 The HIF-1α stability and apoptosis levels were determined by western blot using HIF-1α and caspase 3 antibodies in HepG2 and Hepa1-6 treated with 0, 10, 20 μmol/L Dox. β-Actin, as a loading control. The expression level of shNC cell was considered as 1, and the values for the silenced cells were expressed as ratio of the control cell value.

Materials and methods

HCC cell lines

The human hepatocellular carcinomas cell line HepG2 and mouse hepatoma cell line Hepa1-6 were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Hyclone, Utah, USA) supplemented with 10% fetal bovine serum (FBS, Gemini, CA, USA) in a 37 °C humidified incubator with 5% CO_2.

Knockdown of TRMT5 by short hairpin RNA

To stably knock down TRMT5 in HCC cells, the short hairpin RNA (shRNA) containing a hairpin loop was synthesized and inserted into the pLKO.1-puro vector (Addgene, #8453), and a scramble shRNA was used as the negative control. The sequences are shown in Table S1. For lentivirus production, pLKO.1 with shRNA was co-transfected into HEK293T cells with packaging plasmid psPAX2 (Addgene, #12260) and envelope plasmid pMD2.G (Addgene, #12259) using JetPRIME (Polyplus, Strasbourg, France). 48 h after transfection, the virus was harvested and employed for HCC cell infection. Then, the transfected cells were cultured in the regular medium supplied with 1 μg/mL puromycin for a two-week selection, resulting in homogeneous populations.

Table S1 Sequences of insertions encoding shRNA

shRNA	Oligonucleotides sequence (5'–3')
shNC	CCGGAAGCTTCGCGCCGTAGTCTTACTCGAGTAAGACTACGGCGCGAAGCTTTTTTTG
Sh*TRMT5*_1	CCGGGCAGTTCAGTTCACCTGGTAACTCGAGTTACCAGGTGAACTGAACTGCTTTTTG
Sh*TRMT5*_2	CCGGTGACAATATGTACCGAAATTTCTCGAGAAATTTCGGTACATATTGTCATTTTTG
Sh*Trmt5*-1	CCGGGCCAGTCAGAGAAGAGTTAATCTCGAGATTAACTCTTCTCTGACTGGCTTTTTG
Sh*Trmt5*-2	CCGGCGAGTGATTGAGGATCCCAAACTCGAGTTTGGGATCCTCAATCACTCGTTTTTG

Western blot analysis

Total proteins were extracted using RIPA lysis buffer (Takara, Beijing, China). 20 μg of total proteins were loaded on sodium dodecyl sulfate-polyacrylamide gels and then transferred to a PVDF membrane (Millipore, Massachusetts, USA). The PVDF membranes were incubated with primary antibodies overnight at 4 °C. Antibodies used in this study include TRMT5 (18255-1-AP), GAPDH (60004-1-Ig), EPCAM (21050-1-AP), Vimentin (10366-1-AP), MMP13 (18165-1-AP), VEGFA (66828-1-Ig), PDK1 (10026-1-AP), CASPASE 3 (19677-1-AP), and ACTIN (66009-1-Ig) from Proteintech (Wuhan, China), HIF-1α (A7684) from Abclonal (Wuhan, China). Then, the blots were detected using the ECL system after being probed by peroxidase AffiniPure Goat Anti-Mouse IgG and Goat Anti-Rabbit IgG (Beyotime, Beijing, China) as secondary antibodies. Band intensities were quantified from the 16-bit digital image by densitometry in ImageJ and normalized to GAPDH or ACTIN for each target.2.4 Colony formation assay

HCC cells were seeded in 6-wells plates or 12-wells plates with 500-1000 cells/well and cultured for two weeks. Finally, the colonies were visualized by 1% crystal violet (Yeasen,

Shanghai, China) staining, and colonies with more than 50 cells were counted. All experiments were performed in 3 independent trials.

Cell viability assay

This assay was performed using a CCK8 cell viability kit (Yeasen, Shanghai, China) according to the introduction. Briefly, HCC cells were seeded in 96-well plates (2×10^3 cells/well), and at the indicated time point, 10 μL of CCK8 was added into each well for one hour at 37 ℃. Then, the absorbance was measured at 450 nm by Synergy H1 (BioTek, Vermont, USA).

Flow cytometry

For cell cycle determination, a minimal number of 1×10^6 cells per sample were analyzed. For apoptosis analysis, cells were treated with doxorubicin (MCE, Shanghai, China) for 48 h. The experiments were individually carried out according to the manufacturer's protocols using a NovoCyte flow cytometer (ACEA Biosciences, CA, USA) and analyzed using the NovoExpress software.

Migration and invasion assays

For wound-healing assays, cells in 12-well plates were cultured until they reached 100% confluence, then a wound was made by scraping a 10 μL plastic pipette tip along the bottom of the plate. The wound healing process was recorded for 48 h. For cell migration and invasion assays, 1×10^4 cells were seeded into the upper chamber of transwell filter chambers (Corning, New York, USA) in serum-free culture medium. The lower chamber was filled with completed medium with 20% FBS. After 24h for migration assay and 48h for invasion assay, cells on the lower surface of the membrane were stained with Crystal Violet (Yeasen, Shanghai, China), photographed, and counted in six random fields per group under a light microscope.

RNA isolation and quantitative real-time PCR

Total RNAs were extracted using TRIzol reagent (Takara, Japan) following the manufacturer's protocol and reverse transcribed into cDNA using the PrimeScript™ RT Reagent Kit (Takara, Japan). Quantitative real-time PCR analyses were performed using an Hieff® qPCR SYBR Green Master Mix (Yeasen, China) on the Applied Biosystems Prism 7500 System with gene-specific primers. *GAPDH* was used as the endogenous control. The $2^{-\Delta\Delta CT}$ method was selected for transcript quantification. Detailed primer sequences are provided in Table S2.

Table S2 Sequences of primers for real-time PCR analysis

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
TRMT5	CGTTGATTCCAGTAGCTTGGAC	CTGTTTCTGGCATGGTTGAGA

Trmt5	CTGACGTTCGAGGAATGAGAGA	GCTCGCATCAATCTGTTGACTAC
VIM	GACGCCATCAACACCGAGTT	CTTTGTCGTTGGTTAGCTGGT
Vim	CTGCTTCAAGACTCGGTGGAC	ATCTCCTCCTCGTACAGGTCG
CDH1	CGAGAGCTACACGTTCACGG	GGGTGTCGAGGGAAAAATAGG
Cdh1	TCGGAAGACTCCCGATTCAAA	CGGACGAGGAAACTGGTCTC
CDH2	AGCCAACCTTAACTGAGGAGT	GGCAAGTTGATTGGAGGGATG
Cdh2	AGGCTTCTGGTGAAATTGCAT	GTCCACCTTGAAATCTGCTGG
PDK1	GAGAGCCACTATGGAACACCA	GGAGGTCTCAACACGAGGT
VEGFA	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
EGFR	TTGCCGCAAAGTGTGTAACG	GTCACCCCTAAATGCCACCG
EGLN3	CTGGGCAAATACTACGTCAAGG	GACCATCACCGTTGGGGTT
SERPINE1	GCACCACAGACGCGATCTT	ACCTCTGAAAAGTCCACTTGC
TIMP1	AGAGTGTCTGCGGATACTTCC	CCAACAGTGTAGGTCTTGGTG
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG
Gapdh	TCAACAGCAACTCCCACTCTTCCA	ACCCTGTTGCTGTAGCCGTATTCA

In vivo tumorigenicity assay

Experiments and animal care were performed following the guidelines of Zhejiang University (application ID: 18021). 6~8 weeks old balb/c nude mice were purchased from Shanghai SLAC Animal Inc. 4×10^6 Hepa1-6 cells were suspended in 200 uL of Matrigel and xenografted into the subcutaneous layer of nude mice. The tumor sizes were monitored daily, and tumor volume was calculated using the following formula: volume = $0.52 \times \text{length} \times \text{width}^2$. Mice were sacrificed two weeks after xenograft, and tumor sections were used for histologic examination.

To test tumor metastasis, a total of 2×10^6 Hepa1-6 cells suspended in 200 uL PBS were intravenously injected into nude mice. 8 weeks after injection, mice were sacrificed, the lungs were fixed by Bouin's Fluid (Solabio, Beijing, China)), and tumors in the lungs were quantitated.

RNA sequencing

Total RNAs of HepG2 shNC cells and shRNA1 cells were extracted using TRIzol reagent (Takara, Japan). RNA-seq libraries were constructed, and sequencing was performed with Illumina NovaSeq 6000 sequencer by Novogene Bioinformatics Institute (Beijing, China). Differential gene expression analysis between shNC and sh*TRMT5* cells was performed using the DESeq2 R package (1.20.0). Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the cluster Profiler R package, in which gene length bias was corrected. GO terms with adjusted *p* value less than 0.05 were considered significantly enriched by differential expressed genes. Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis was performed using Cluster Profiler R package.

Measurement of ECAR and OCR

Cellular oxidative phosphorylation and glycolysis alternations were determined with the Seahorse XF96 Flux Analyzer (Seahorse, Agilent, CA, USA) by measuring the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) respectively in real-time according to the manufacturer's instructions.

ATP measurement

The cellular ATP levels were measured by Enhanced ATP Assay Kit (Beyotime, China) according to the manufacturer's instructions.

Lactate measurement

The lactate concentrations were determined by LA Assay Kit (Solarbio, China) according to the manufacturer's instructions.

Immunofluorescence staining

Immunofluorescence staining was assayed as previously described (Mori and Cardiff, 2016). Antibodies were used including Ki67 (CST, #9129S, Boston, USA), and Hypoxyprobe Kit (HPI, Massachusetts, USA).

Statistical analysis

Statistical analysis was carried out based on at least three independent experiments using unpaired two-tailed Student's t-test and two-way ANOVA in the GraphPad Prism 8 program. The error bars indicate two standard deviations of the means. Differences were considered significant at a *p* value of <0.05.

Reference

Mori H, Cardiff RD, 2016. Methods of immunohistochemistry and immunofluorescence: Converting invisible to visible. *Methods Mol Biol*, 1458:1-12. https://doi.org/10.1007/978-1-4939-3801-8_1