

LTBP4 Inhibits the Proliferation and Metastasis in Melanoma by Activating Hippo-YAP Signaling

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Research

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Abstract

Background: Malignant melanoma is the deadliest of skin cancer. The present study aimed to elucidate potential key candidate genes in melanoma and its molecular mechanism.

Methods: Three gene expression profile data sets (GSE46517, GSE52882 and GSE54493) were downloaded from the GEO database, which included data from melanoma tissue samples and cell lines. DEGs were subsequently investigated by GO analysis via using DAVID website. PPI network was constructed using the STRING database and visualized by Cytoscape software and MCODE were utilized to PPI network to pick out meaningful DEGs. Cell proliferation, apoptosis, migration and invasion were measured using CCK-8, colony formation, flow cytometry, transwell and wound healing assays. RT-PCR, western blotting and immunohistochemistry assays were used to detect mRNA and protein expressions. TCGAportal and GEPIA databases were used to perform the bioinformatics analysis of LTBP4 in melanoma.

Results: LTBP4 both is the DEG and a key gene from the most significant module of the PPI network. LTBP4 expression was down-regulation in melanoma tissues and cells relative to controls, which showed positive correlation with invasion, TNM stage, distal metastasis and lymph node metastasis, and predicted the poor prognosis for patients with melanoma. Cox analysis identified LTBP4 low-expression as an independent prognostic variable for overall survival (OS) in patients with melanoma. The results revealed that LTBP4 inhibition reduced cell apoptosis, promoted cell proliferation and metastasis. These changes were correlated caspase-3, ki67 and E-cadherin expressions by western blotting assay. Further *in vivo* tumor formation study in nude mice indicated that LTBP4 inhibition promoted the progress of tumor formation. LTBP4 gene knockout reduced the phosphorylation level of YAP, MST1 and MOB1 and promoted the nuclear translocation of YAP to inhibit the activation of Hippo signaling pathway. The functions of LTBP4 overexpression (OE) inhibiting the expressions of CTGF, Cyr61 and Birc5, promoting the apoptosis, and inhibiting the metastasis and proliferation of melanoma cells were reversed by YAP/or MST1 OE.

Conclusions: LTBP4 OE suppressed the proliferation and metastasis in melanoma via inhibiting the nuclear translocation of YAP to activating Hippo signaling pathway, thereby inhibiting the development and progression of melanoma.

Background

Melanoma is a malignant tumor that originates from melanocytes in the skin and other tissues, accounting for more than 75% of all skin cancers [1]. Malignant melanoma is the most invasive skin cancer in the world, which is produced by melanocytes. As we all know, surgical resection is an effective treatment for early melanoma, but many advanced patients with melanoma can't accept surgical treatment, usually with surgery combined with adjuvant chemotherapy [2]. Chemotherapy resistance and high metastasis lead to the poor prognosis in patients with advanced melanoma [3], therefore, it is very

important to study the pathogenesis of melanoma and find effective potential targets for the diagnosis and treatment of melanoma.

Hippo signal pathway is involved in the regulation of many kinds of cell and organ homeostasis, and mainly composed of a series of kinase complexes and downstream transcriptional coactivators, including MST1/2, SAV1, LATS1/2, MOB1 and YAP/TAZ [4-6]. Activated Hippo signal pathway induces the binding of MST1 kinase and its junction protein SAV1, thereby activating LATS1/2 and MOB1 kinase. Activated LATS1/2 and MOB1 kinases further promote the phosphorylation of YAP protein [7]. Phosphorylated YAP cannot translocate from the cytoplasm to the nucleus, thus leaving it trapped in the cytoplasm. However, most of the phosphorylated YAP in the cytoplasm were degraded by ubiquitin. Inactivated Hippo pathway rarely could make phosphorylation to YAP protein. The unphosphorylated YAP protein enriches in the nucleus via translocating into the nucleus to promote the expression of related genes that contribute to cell proliferation and survival [8]. Therefore, the activation of Hippo pathway determines the phosphorylation level and subcellular localization of YAP protein, which also determines the role of this pathway in tumorigenesis and development.

Hippo pathway is in a dynamic balance to maintain cell proliferation, apoptosis and the stability of the internal environment of the body. It was reported that inactivated Hippo pathway decreases YAP phosphorylation and promotes YAP nuclear translocation in many kinds of tumors [9-11]. For example, inactivated Hippo signal pathway or YAP overexpression (OE) caused abnormal liver development and eventually led to the occurrence of liver cancer in mice [12]; SOX2 OE promoted the proliferation and migration of esophageal squamous cell carcinoma cells by inhibiting the activation of Hippo signal pathway, and improve the sensitivity of cells to cisplatin [13]. In addition, YAP OE promoted the proliferation and metastasis and inhibit the apoptosis in A375 cells [14]. It is suggested that Hippo signaling pathway played an important role in the occurrence and development of melanoma.

Methods

Melanoma cancer database and data processing

GSE46517, GSE52882 and GSE54493 datasets containing mRNA expression profiles were downloaded from the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>). GSE46517 dataset included 5 normal tissue samples, 5 primary melanoma tissue samples and 5 metastatic melanoma tissue samples; GSE52882 included 3 DMSO treated A375 cells, 3 vemurafenib treated A375 cells, 3 DMSO treated A2058 cells and 3 vemurafenib treated A2058 cells; GSE54493 included 2 mock transfected SK-Mel-147 cells, 2 miR-638 OE transfected SK-Mel-147 cells, and 2 miR-638 knockdown (KD) transfected SK-Mel-147 cells.

Identification of DEGs

Firstly, the experiment was divided into six groups: 1) 5 normal tissue samples vs 5 primary melanoma tissue samples in GSE46517 dataset ; 2) 5 normal tissue samples vs 5 metastatic melanoma tissue samples in GSE46517 dataset ; 3) 3 DMSO treated A375 cells vs 3 vemurafenib treated A375 cells in GSE52882 dataset; 4) 3 DMSO treated A2058 cells and 3 vemurafenib treated A2058 cells in GSE52882 dataset; 5) 2 mock transfected SK-Mel-147 cells vs 2 miR-638 OE transfected SK-Mel-147 cells in GSE54493 dataset; 6) mock transfected SK-Mel-147 cells vs 2 miR-638 KD transfected SK-Mel-147 cells in GSE54493 dataset. The DEGs were calculated using the “GEO2R” online tool (<http://www.ncbi.nlm.nih.gov/geo/geo2r>). The DEGs of the database with an absolute Log₂ fold change (FC) > 1 and *P* value < 0.05 were considered for subsequent analysis.

Functional enrichment analysis

The GO enrichment was used to analyze DEGs at the functional level with DAVID (<http://david.ncifcrf.gov/>, version 6.8). DAVID is a comprehensive database of functional annotation tools for connecting functional terms with gene lists using a clustering algorithm. In order to elucidate the functional profiles of the DEGs, we used DAVID to obtain the enriched biological process (BP), cellular component (CC), molecular function (MF) and KEGG pathway. *P* < 0.05 was considered statistically significant.

PPI network analysis

The STRING database (<http://string-db.org/>) was used to identify the interactions between proteins encoded by DEGs based on experimental data, databases, text mining, and predictive bioinformatics data. A combined score of > 0.4 was set as threshold value. PPI networks were constructed with Cytoscape software. The plug-in MCODE was used to screen the modules of PPI network in Cytoscape software. MCODE score ≥ 3 and number of nodes ≥ 3 were considered as threshold value.

Expression and survival analysis of IFITM1 and LTBP4

TCGAportal (<http://www.tcgaportal.org>) and GEPIA (<http://gepia.cancer-pku.cn/>) were used to investigate survival probability and the expression of IFITM1 and LTBP4 in tumor tissues and corresponding paracarcinoma tissues.

Cells and surgical specimens

Seven melanoma cell lines used in this study. A101D, 451Lu, SK-MEL-1, VMM5A, G-361, A375, and MeWo cells and control cell (HaCaT cell) were purchased from the Cell Culture Collection of the Chinese Academy of Sciences (Beijing, China) and cultured in DMEM medium containing 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 mg/mL streptomycin, and 100 U/mL penicillin in a humidified incubator

containing 5% CO₂ at 37°C for 2-3 days. Specimens of melanoma tissues and adjacent noncancerous tissues were collected from 76 patients with melanoma between October 2018 and October 2019, following surgical resection at Tianjin Medical University Eye Hospital. All experiments were approved by the Medical Ethics Committee of Tianjin Medical University Eye Hospital and written informed consent documents were signed by all of the patients. Table 1 lists the clinical characteristics of the enrolled patients.

LTBP4 gene silencing and OE and cell transfection

Two different shRNAs (shRNA-1 and shRNA-2; purchased from GeneChem, Shanghai, China) were used to target the LTBP4 gene KD. A nonsilencing shRNA (NC) was used as control (GeneChem). LTBP4 expressing plasmids (constructed using a pcDNA 3.1 vector) (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) to induce LTBP4 OE. Cells were transfected with 10 nM of shRNAs or pcDNA3.1 when cells reached 30-50% confluence. After 8 hours of transfection using Lipofectamine-2000 (Life Technologies) according to the manufacturer's protocol, cells were returned to normal medium in the incubator. When cells reached 85% confluence, cell supernatant, protein, and RNA were collected for later experiments.

YAP gene or MST1 genes OE and cell transfection

YAP expressing plasmids or MST1 expressing plasmids (constructed using a pcDNA 3.1 vector) (Invitrogen; Thermo Fisher Scientific, Inc) to induce YAP OE or MST1 OE. SK-MEL-1 and A375 cells were transfected with 10 nM of pcDNA3.1 when cells reached 30-50% confluence. After 8 hours of transfection using Lipofectamine-2000 (Life Technologies) according to the manufacturer's protocol.

Rescue experiment

This experiment was divided into the following four groups: 1) SK-MEL-1 or A375 cells without any treatment was viewed as CTRL group; 2) SK-MEL-1 or A375 cells transfected with LTBP4 OE was viewed as LTBP4-OE group; 3) SK-MEL-1 or A375 cells co-transfected with LTBP4 OE and YAP OE was viewed as LTBP4-OE+YAP-OE group; 4) SK-MEL-1 or A375 cells co-transfected with LTBP4 OE and MST1 OE was viewed as LTBP4-OE+MST1-OE group. Cell proliferation, apoptosis, migration and invasion in above groups were measured using CCK-8, colony formation, flow cytometry, transwell and wound healing assays.

Cell viability assay

Cells were transfected with shRNAs or pcDNA3.1 for 48 h to detect cell viability according to CCK-8 kit instructions (Beyotime, Shanghai, China). Transfected cells (5,000 cells per well) were incubated in a cell

incubator for 0.5-4 h and tested at 0, 6, 12, 24, 48 and 72 h, respectively, using a microplate reader. Absorbance was measured at 450 nm.

Colony Formation

For the colony formation assay, 600 cells per group were plated in triplicates in a six-well plate. Cells were allowed to grow till visible colonies appeared, stained with crystal violet (Beyotime), washed with PBS, and counted.

Flow cytometry analysis

For apoptosis measurements, the percentages of apoptotic cells were determined by flow cytometry using the Annexin V-FITC/PI cell apoptosis detection kit (Promega) according to the manufacturer's instructions.

Wound-healing assay

After transfection with the different plasmids for 24 h, cells were seeded in a 24-well plate and grown to confluence. The confluent monolayer of cells was wounded using a standard 200 μ L pipette tip and then washed three times to remove the non-adherent cells. Wounds were monitored and photographed at the time the scrape was created and 24 h later. Cell migration capacity was calculated according to the width of the wounds at 0 h and 24 h. The migration rate is described as a percentage of the migration observed in the control group under an inverted microscope (Olympus).

Transwell invasion assays

Transwell assays were conducted to detect the invasion capabilities of cells. Cells (4×10^5 /mL) were plated within the top chamber coated with Matrigel membrane. FBS 10% was used as a chemoattractant in the lower chamber. Cells were incubated for 48 hours, and the cells that did not invade through the membrane were removed by a cotton swab. Cells on the lower chamber were then stained with crystal violet and counted under an inverted microscope (Olympus).

In vivo tumorigenicity

Animal experiments were done according to Institutional Animal Care and Use Committee (IACUC) protocol and approved by Tianjin Medical University Animal Center for Use and Care of Animals. The establishment of the subcutaneous melanoma tumor model was treated by subcutaneous injection of SK-MEL-1 cells. A total of 12 4-week-old female BALB/C nude mice (Tianjin Medical University Animal

Center, Tianjin, China) were required to establish melanoma model mice for 4 weeks. Mice were sacrificed 30 days after the injection and size of the tumor was measured by vernier caliper and weight of the tumor was measured by electronic balance.

Immunohistochemistry (IHC) staining

LTBP4 expression between melanoma tissues and adjacent noncancerous tissues in patients with melanoma was determined by IHC staining. For IHC, sections were incubated with anti-LTBP4 (1:300 dilution) antibody. LTBP4 staining was scored by two independent pathologists. LTBP4 or cleaved caspase-3 expression between NC group and LTBP4-KD group in tumor tissues of BALB/C nude mice was determined by IHC staining. For IHC, sections were incubated with anti-LTBP4 (1:500 dilution) antibody or anti-cleaved caspase-3 (1:200 dilution) antibody. LTBP4 or cleaved caspase-3 staining was scored by researcher. The scoring system was based on the staining intensity and extent. Staining intensity was classified as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Staining extent depended on the percentage of positive cells and was divided into 0 (<5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), and 4 (>75%). According to the staining intensity and the staining extent scores, the IHC result was classified as 0–1, negative (-); 2–4, weakly positive (+); 5–8, moderately positive (++); and 9–12, strongly positive (+++).

Western blotting

Protein lysates were prepared from cells using 500 μ L of RIPA buffer with 1 mM phenylmethane sulfonyl fluoride. Samples were subsequently sonicated for 2 minutes and centrifuged. The supernatants were collected and used for protein analysis. Lysates were separated on 8% polyacrylamide gels and transferred onto polyvinylidene fluoride membrane. The membranes were blocked with PBS containing 0.1% Tween-20 (PBST) and 5% nonfat milk (w/v) for 1 hour at room temperature. After they were washed with PBST, the membranes were probed with antibodies overnight at 4°C. Antibody against LTBP4 was obtained from Shanghai Yu Bo Biotech Co.,Ltd (Shanghai, China); antibody against cleaved caspase-3/Ki67/E-cadherin/YAP/MST1/CTGF/Cyr61 and phosph-YAP/MST1 antibody was obtained from Abcam (Cambridge, UK); antibody against MOB1 and phospho-MOB1 were obtained from Cell Signaling Technology, Inc (USA); antibody against Birc5 and phospho-Birc5 were obtained from CUSABIO engineering co. Ltd (Wuhan, Hubei, China); antibody against β -actin or LaminB were obtained from Beyotime (China). The membranes were washed again with PBST, then horseradish peroxidase-labeled IgG at 1:5,000 dilution was added at room temperature for 1 h, and the blots were developed using enhanced chemiluminescence western blotting reagents. β -actin or LaminB was used as an internal control.

Real-time PCR (RT-PCR)

Real-time PCR was performed on a Step Two Real-Time PCR System (Applied Biosystems) using the comparative C_t quantization method. Real-time PCR Master Mix (Toyobo) was used to detect and quantify the expression level of the target gene. GAPDH was used as an internal control. The primers used were as follows: CTGF, 5'- AGTGCATCCGTA CTCCCAA-3' (F) and 5'- CCGTCGGTACATACTCCACA-3' (R); Cyr61, 5'- GCAGCGTTTCCCTTCTACAG-3' (F) and 5'- ATGAGTCCCATCACCCACAC-3' (R); Birc5, 5'- AACAGTGGCTGCTTCTCTCT-3' (F) and 5'- GCCTTCTTCTCCCTCACTT-3' (R); β -actin, 5'- ACTCTTCCAGCCTTCCTTCC-3' (F), 5'-CAATGCCAGGGTACATGGTG-3' (R); YAP, 5'- TCCACCAGTGCAGCAGAATA-3' (F) and 5'- TTGGGTCTAGCCAAGAGGTG-3' (R); MST1, 5'- TCCCAGTAGCCAAGATGGTG-3' (F) and 5'- TGGAGGCACCACATACCATT-3' (R); LTBP4, 5'- CGACATGCCAGACTTTGAGG-3' (F) and 5'- ACCAGCATAGCTTCCACCTT-3' (R).

Statistical analysis

Statistical analyses were performed using SPSS 19.0 (IBM, Armonk, NY, USA). Correlations between LTBP4 expression and the clinicopathological variables were analyzed using the Pearson χ^2 analysis. The average value of LTBP4 > 1.925 as high expression. Survival was analyzed using the Kaplan-Meier method, and differences were evaluated using the log-rank test. The Cox proportional hazards model was used for univariate analysis to examine the potential prognostic value of different variables on OS. Data were evaluated using ANOVA with LSD test for multiple comparisons and Student's *t* test between two groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Bioinformatics analysis of DEGs

There were 6791 DEGs (2695 downregulated and 4096 upregulated) between 5 normal tissue samples and 5 primary melanoma tissue samples in GSE46517 database. There were 4020 DEGs (1739 downregulated and 2281 upregulated) between 5 normal tissue samples and 5 metastatic melanoma tissue samples in GSE46517 database. There were 3340 DEGs (1429 downregulated and 1911 upregulated) between 3 DMSO treated A375 cells and 3 vemurafenib treated A375 cells in GSE52882 dataset. There were 2436 DEGs (1004 downregulated and 1432 upregulated) between 3 DMSO treated A2058 cells and 3 vemurafenib treated A2058 cells in GSE52882 dataset. There were 7422 DEGs (3220 downregulated and 4202 upregulated) between 2 mock transfected SK-Mel-147 cells and 2 miR-638 OE transfected SK-Mel-147 cells in GSE54493 dataset. There were 7979 DEGs (3372 downregulated and 4607 upregulated) between 2 mock transfected SK-Mel-147 cells and 2 miR-638 KD transfected SK-Mel-147 cells in GSE54493 dataset (Fig. 1a). A total of 54 overlapping DEGs from GSE46517, GSE52882 and GSE54493 datasets were identified by Venn analysis (Fig. 1b). In addition, the expression of 54 overlapping genes in GSE46517, GSE52882 and GSE54493 datasets was shown in Fig. 1c. IFTM1 and LTBP3 were key genes from significant modules of the PPI network of 54 overlapping genes was identified using STRING database and Cytoscape software (Fig. 1d). There was no significant difference

for in IFTM1 or LTBP3 at survival probability between high expression group and low expression group, however, LTBP4 was significantly downregulation in melanoma tissues based on GEPIA database analysis (Fig. 1e). GO enrichment analysis showed that 54 overlapping DEGs mainly enriched in BP and CC terms via using DAVID database and LTBP4 mainly enriched in “extracellular matrix” (Fig. 1f and 1 g).

LTBP4 expression was down-regulation in melanoma tissues and cell lines, which was closely related to the poor survival for patients with melanoma

The results of RT-PCR and western blotting assays showed that LTBP4 were significantly down-regulated in cancer tissues of 76 patients with melanoma, which were associated with invasion, TNM stage, distal metastasis and lymph node metastasis (Fig. 2a-b and Table 1). In addition, 76 patients with melanoma were divided into LTBP4 high-expression and LTBP4 low-expression groups according to the average value of LTBP4 expression (average value = 1.925). Kaplan-Meier curve was performed to estimate survival, and log-rank test was used for comparison between the curves. The OS of the LTBP4 high-expression group was longer (mean OS: 1044.0 days, 95% CI: 979.99-1108.01 days) than that of the LTBP4 low-expression group (mean OS: 895.9 days, 95% CI: 756.99-1034.82 days) ($P=0.007$) (Fig. 2c). Univariate Cox regression analysis revealed that low LTBP4 expression [hazard ratio (HR), 0.062; 95% confidence interval (CI), 0.006–0.694; $P=0.024$] was associated with survival (Table 2). Invasion, TNM stage, distal metastasis, lymph node metastasis, age and sex were not associated with survival (all $P>0.05$). Likewise, LTBP4 expression at protein level was significantly down-regulation in melanoma tissues via using IHC staining (Fig. 2d and 2e). Next, we found that LTBP4 expression was significantly downregulation in melanoma cell lines via using RT-PCR and western blotting assay (Fig. 2f and 2 g). LTBP4 expression in SK-MEL-1 and VMM5A cells was significantly higher than that in A101D and A375 cells. After shRNA-LTBP4 #1 or #2 was transfected into SK-MEL-1 and VMM5A cells to inhibit LTBP4 expression and LTBP4 expressing plasmids were transfected into A101D and A375 cells to overexpress LTBP4 expression (Fig. 2h and 2i).

Table 1

Correlation between LTBP4 expression and clinicopathological parameters of patients with melanoma

Parameters	Number of patients		χ^2	<i>P</i> -value
	Low LTBP4 expression (n)	High LTBP4 expression (n)		
Age (years)			0.015	0.901
<60	25	15		
≥60	22	14		
Sex			0.926	0.336
Male	28	14		
Female	19	15		
Invasion			11.135	0.001*
T0-T2	14	20		
T3-T4	33	9		
TNM stage			13.879	0.000*
I-II	12	20		
III-IV	35	9		
Distal metastasis			19.418	0.000*
Yes	37	8		
No	10	21		
Lymph node metastasis			9.909	0.002*
Yes	32	9		
No	15	20		
TNM stage: Tumor-Lymph Node-Metastasis stage; Latent transforming growth factor beta binding protein 4 (LTBP4); * <i>P</i> <0.05 was considered as significant.				

Table 2

Univariate Cox proportional hazards analyses of LTBP4 expression and overall survival for patients with melanoma

Univariate analysis		
	HR (95% CI)	P-value
Invasion T0-T2 vs T3-T3	0.799 (0.228–2.797)	0.726
TNM stage I-II vs III-IV	0.650 (0.165–2.556)	0.537
Distal metastasis Yes vs No	1.278 (0.342–4.781)	0.715
Lymph node metastasis Yes vs No	1.176(0.365–3.790)	0.786
Age < 60 vs ≥ 60	1.406(0.434–4.550)	0.570
Sex Male vs Female	1.013(0.303–3.390)	0.983
LTBP4 expression Low vs High	0.062 (0.006–0.694)	0.024*
TNM stage: Tumor-Lymph Node-Metastasis stage; Latent transforming growth factor beta binding protein 4 (LTBP4);		
HR, Hazard ratio; CI, confidence interval. * $P < 0.05$ was considered as significant.		

LTBP4 significantly regulated the proliferation and apoptosis in melanoma cell lines

Our results showed that LTBP4 KD promoted the viability of SK-MEL-1 and VMM5A cells (Fig. 3a and 3b), whereas LTBP4 OE inhibited the viability of A101D and A375 cells (Fig. 3c and 3d). Not surprisingly, LTBP4 KD promoted the proliferation of SK-MEL-1 and VMM5A cells (Fig. 3e), and LTBP4 OE inhibited the proliferation of A101D and A375 cells (Fig. 3f). Flow cytometry results showed that the apoptosis level in SK-MEL-1 and VMM5A cells was significantly suppressed by LTBP4-KD1 or LTBP4-KD2, meanwhile, the apoptosis level in A101D and A375 cells was significantly promoted by LTBP4 OE (Fig. 3g and 3 h). However, there was no significant differences in cell viability, proliferation and apoptosis between LTBP4-KD1 group and LTBP4-KD2 group.

LTBP4 significantly regulated the invasion and migration in melanoma cell lines, and closely related to the expressions of cleaved caspase-3, Ki67 and E-cadherin

Transwell assay results showed that silencing of LTBP4 significantly promoted the invasion of SK-MEL-1 and VMM5A cells (Fig. 4a) and OE of LTBP4 markedly restrained the invasion of A101D and A375 cells (Fig. 4b). Conversely, wound healing assay results showed that silencing of LTBP4 significantly promoted the migration of SK-MEL-1 and VMM5A cells (Fig. 4c) and OE of LTBP4 markedly restrained the migration of A101D and A375 cells (Fig. 4d). Next, the changes of cleaved caspase-3, Ki67 and E-cadherin expression in LTBP4 KD or OE transfected melanoma cell lines were determined via using western blotting. The results showed that silencing of LTBP4 significantly inhibited the expressions of cleaved caspase-3 and E-cadherin, and increased Ki67 expression in SK-MEL-1 and VMM5A cells, meanwhile, OE of LTBP4 significantly increased the expressions of cleaved caspase-3 and E-cadherin and suppressed Ki67 expression in A101D and A375 cells (Fig. 4e and 4f).

Silencing of LTBP4 promoted the oncogenicity of melanoma cell in vivo

After shRNA-LTBP4 transfected SK-MEL-1 cells were injected into the nude mice subcutaneously, tumor weight and volume were measured via using electronic balance and vernier caliper (Fig. 5a). LTBP4 KD promoted tumor volume and weight (Fig. 5b and 5c). IHC staining results showed that the expressions of LTBP4 and cleaved caspase-3 were inhibited by LTBP4 KD and the expressions of Ki67 and E-cadherin were increased by LTBP4 KD (Fig. 5d and 5e).

Silencing of LTBP4 suppressed the activation of Hippo signaling pathway via inhibiting the phosphorylation of YAP to promote the nuclear translocation of YAP, thereby promoting the expressions of CTGF, Cyr61 and Birc5 in SK-MEL-1 and VMM5A cells

Our results showed that silencing of LTBP4 upregulated the expressions of YAP, MST1, and MOB1 and suppressed the phosphorylation of YAP, MST1, and MOB1 in SK-MEL-1 and VMM5A cells via using western blotting (Fig. 6a and 6b). Next, the mRNA and protein levels of CTGF, Cyr61, and Birc5 in shRNA-LTBP4 transfected SK-MEL-1 and VMM5A cells were significantly upregulation via using RT-PCR and western blotting assays (Fig. 6c and 6d). Furthermore, the low expression of YAP was found in cytoplasm of shRNA-LTBP4 transfected SK-MEL-1 and VMM5A cells (Fig. 6e), meanwhile, the high expression of YAP was found in nucleus of shRNA-LTBP4 transfected SK-MEL-1 and VMM5A cells (Fig. 6f). In addition,

there was no significant differences for the expressions of YAP, MST1, MOB1, CTGF, Cyr61, and Birc5 in SK-MEL-1 and VMM5A cells between LTBP4-KD1 group and LTBP4-KD2 group.

OE of LTBP4 activated Hippo signaling pathway via promoting the phosphorylation of YAP to inhibit the nuclear translocation of YAP, thereby inhibiting the expressions of CTGF, Cyr61 and Birc5 in A101D and A375 cells

Our results showed that OE of LTBP4 downregulated the expressions of YAP, MST1, and MOB1 and promoted the phosphorylation of YAP, MST1/2, and MOB1 in A101D and A375 cells via using western blotting (Fig. 7a and 7b). Next, the mRNA and protein levels of CTGF, Cyr61, and Birc5 in LTBP4 expressing plasmids transfected A101D and A375 cells were significantly downregulation via using RT-PCR and western blotting assays (Fig. 7c and 7d). Furthermore, the high expression of YAP was found in cytoplasm of LTBP4 plasmids transfected A101D and A375 cells (Fig. 7e), meanwhile, the low expression of YAP was found in nucleus of LTBP4 plasmids transfected A101D and A375 cells (Fig. 7f).

OE of LTBP4 inhibited the proliferation, invasion and migration, and promoted the apoptosis of melanoma cells via Hippo-YAP signaling

YAP/or MST1 expressing plasmids were transfected into SK-MEL-1 and A375 cells to induce YAP/or MST1 OE via using RT-PCR and western blotting assays (Fig. 8a and 8b). Furthermore, the results of CCK-8 and colony formation assays indicated that the functions of LTBP4 OE inhibiting the viability and proliferation of SK-MEL-1 and A375 cells were reversed by YAP OE or MST1 OE (Fig. 8c and 8d). In addition, YAP OE or MST1 OE significantly abolished the effects of LTBP4 OE on promoting the apoptosis of SK-MEL-1 and A375 cells (Fig. 8e). The functions of LTBP4 OE inhibiting the invasion and migration of SK-MEL-1 and A375 cells were reversed by YAP OE or MST1/2 OE (Fig. 8f and 8g). Similarly, western blotting results showed that the functions of LTBP4 OE increased the cleaved caspase-3 and E-cadherin expression and reduced Ki67 expression counteracted by YAP OE or MST1 OE in SK-MEL-1 and A375 cells (Fig. 9a-9d). Next, both YAP OE and MST1 OE could inhibit the functions of LTBP4 OE decreasing CTGF, Cyr61, and Birc5 in SK-MEL-1 and A375 cells (Fig. 9e-9h).

Discussion

Our results showed that LTBP4 is a key gene in melanoma based on bioinformatics analysis (Fig. 1). It is reported that LTBP is a class of extracellular matrix glycoproteins belonging to the fibrillin superfamily,

mainly including four subtypes of LTBP1, LTBP2, LTBP3 and LTBP4 [15–18]. LTBP family regulated the occurrence and development of cancer via binding to TGF- β [19]. LTBP4 expression might be linked to the carcinogenesis of human mammary carcinomas, ductal carcinoma in situ and mouse mammary tumors and DCIS [20]. In addition, LTBP4 is downregulated in human mammary adenocarcinomas and may be involved in neoplastic transformation of human mammary tumors [20, 21]. Disruption of the gene encoding the LTBP4 causes abnormal lung development, cardiomyopathy, and colorectal cancer [22]. However, the study of LTBP4 in malignant melanoma has not been reported. Our results showed that downregulation of LTBP4 was found in melanoma tissues and cell lines and had a poor survival in patients with melanoma (Fig. 2, Table 1 and Table 2). Therefore, we speculated that LTBP4 might be a tumor suppressor gene in melanoma. Furthermore, the results of cell functional experiments indicated that silencing of LTBP4 promoted the viability, proliferation, invasion and migration, and inhibited the apoptosis in melanoma cells, whereas OE of LTBP4 inhibited the viability, proliferation, invasion and migration, and induced the apoptosis in melanoma cells (Fig. 3 and Fig. 4).

Caspase-3 and Ki67 are important proteins related to proliferation and apoptosis, which are abnormally expressed in many kinds of malignant tumors [23]. E-cadherin is an important tumor suppressor gene related tumor metastasis, and its expression is significantly down-regulated in malignant tumor cells with high ability of migration and invasion [24, 25]. In this study, we found that LTBP4 KD significantly inhibited the expression of cleaved caspase-3 and E-cadherin and up-regulated the expression of Ki67 in melanoma cells. Unsurprisingly, the effect of LTBP4 OE on the expression of cleaved caspase-3, E-cadherin and Ki67 in melanoma cells was opposite to that of LTBP4 KD on the expression of cleaved caspase-3, E-cadherin and Ki67 in melanoma cells. By virtue of nude mouse tumorigenesis test, the tumorigenesis ability *in vivo* of LTBP4 KD was further confirmed (Fig. 5). To sum up, we can confirm that LTBP4 gene knockout promotes the growth and metastasis of melanoma and affects the survival status of patients. However, the molecular mechanism of the inhibitory effect of LTBP4 on melanoma needed to be further confirmed.

YAP, as a tumor-promoting protein, is one of the core molecules of Hippo signaling pathway, and highly expressed in almost all melanoma tumor cells [26]. YAP gene knockout can delay wound healing and reduce the expression of TGF- β 1. In addition, YAP regulates TGF- β signaling pathway [27]. It is reported that there is a molecular interaction between LTBP4 and TGF- β RII, and LTBP4 gene knockout leads to a decrease in the number of TGF- β RII [28]. Studies have shown that the decrease and distribution of TGF- β RII protein results in the inability of TGF- β 1 to bind to its receptor, which in turn leads to the inability of TGF- β 1 to inhibit the growth of tumor cells [29]. These reports confirmed laterally that LTBP4 gene knockout promotes the growth of tumor cells. Therefore, we speculated that there was an interaction between LTBP4 and YAP, which was closely related to the activation of Hippo signal pathway. The results showed that LTBP4 KD upregulated the expressions of YAP, MST1, MOB1, and the downstream gene-expression of YAP (CTGF, Cyr61 and Birc5), and inhibited the phosphorylation levels of YAP, MST1, and MOB1 in melanoma cell. Furthermore, LTBP4 KD promoted the nuclear translocation of YAP (Fig. 6). On the contrary, LTBP4 OE downregulated the expressions of YAP, MST1, MOB1, and the downstream gene-expression of YAP (CTGF, Cyr61 and Birc5) [30], and increased the phosphorylation levels of YAP, MST1,

and MOB1 in melanoma cell. Furthermore, LTBP4 OE suppressed the nuclear translocation of YAP (Fig. 7). It showed that LTBP4 KD inhibited the activation of Hippo-YAP signaling in melanoma cells. Rescue experiment results showed that the functions of LTBP4 OE inhibiting the viability, proliferation, invasion, and migration, and promoting the apoptosis in melanoma cells were reversed by YAP OE or MST1 OE (the core molecule of Hippo signaling pathway), meanwhile, the functions of LTBP4 OE inhibiting the expressions of cleaved caspase-3 and E-cadherin, and inhibiting the expressions of Ki67, CTGF, Cyr61 and Birc5 in melanoma cells were abolished by YAP OE or MST1 OE (Fig. 8 and Fig. 9). These results showed that LTBP4 affected the activation of Hippo signaling pathway via regulating the nuclear translocation of YAP.

Conclusions

The downregulation of LTBP4 was found in melanoma tissues and cell lines and had a poor survival in patients with melanoma. Silencing of LTBP4 promoted the growth and metastasis of melanoma *in vivo* and *in vitro*. LTBP4 knockdown suppressed the activation of Hippo signaling pathway via inhibiting the phosphorylation of YAP to promote the nuclear translocation of YAP, thereby promoting the proliferation, invasion and migration, and inhibiting the apoptosis of melanoma cells. These results also raise the possibility that LTBP4 may function as an important new biomarker for melanoma tumors.

Abbreviations

DAVID: Database for Annotation, Visualization and Integrated Discovery

PPI network: Protein-protein interaction network

STRING: Search Tool for the Retrieval Interacting Genes

MCODE: Molecular Complex Detection

GEO database: Gene Expression Omnibus database

LTBP4: Latent transforming growth factor beta binding protein 4

DEG: Differentially expression gene

OS: Overall survival

OE: Overexpression

KD: Knockdown

YAP: Yes1-associated transcriptional regulator

MST1: Macrophage stimulating 1

MOB1: Mps one binder protein 1

CTGF: Connective tissue growth factor

Birc5: Baculoviral IAP repeat containing 5

TGF- β : Transforming growth factor- β

TGF- β RII: Transforming growth factor- β receptor II

GO: Gene Ontology

DCIS: Ductal Carcinoma In Situ

Declarations

Availability of data and materials

The datasets generated/analyzed during the current study are available.

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Contributions

Lina Wang wrote the main manuscript and analyzed the data. Lina Wang, Dongrun Tang and Tong Wu performed the experiments. Lina Wang and Fengyuan Sun designed the study. All authors read and approved the final manuscript.

Corresponding author

Correspondence to Fengyuan Sun.

Ethics declarations

Ethics approval

This study was approved by the Ethics Committee of Tianjin Medical University Eye Hospital. The written informed consent documents were signed by all of the patients.

Consent for publication

All authors approve of the submitted manuscript.

Competing interests

The authors declare that they have no competing interests.

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Supplemental

Supplemental figure 1, figure 3 and figure 5 are not available with this version

Figures

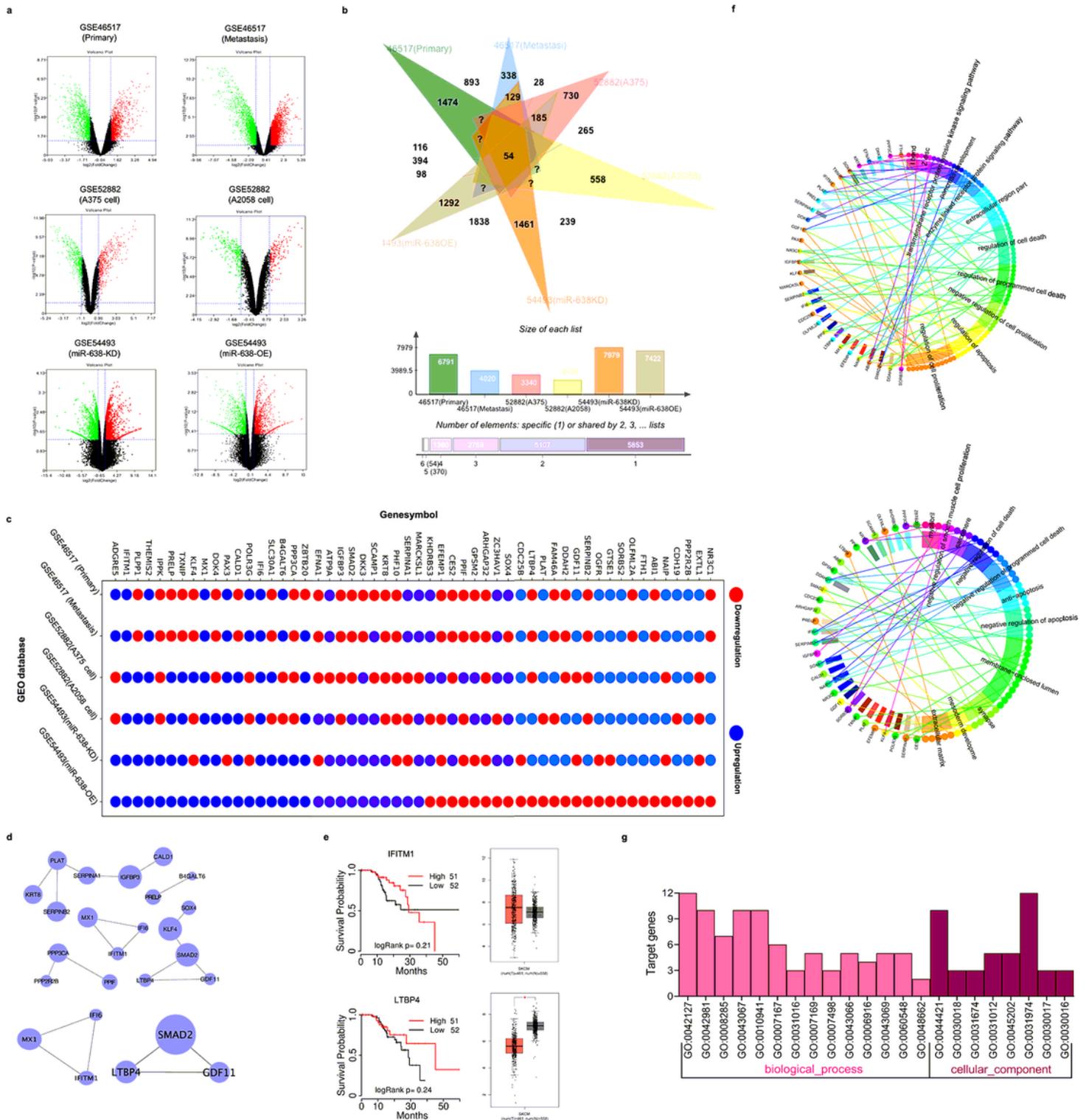


Figure 1

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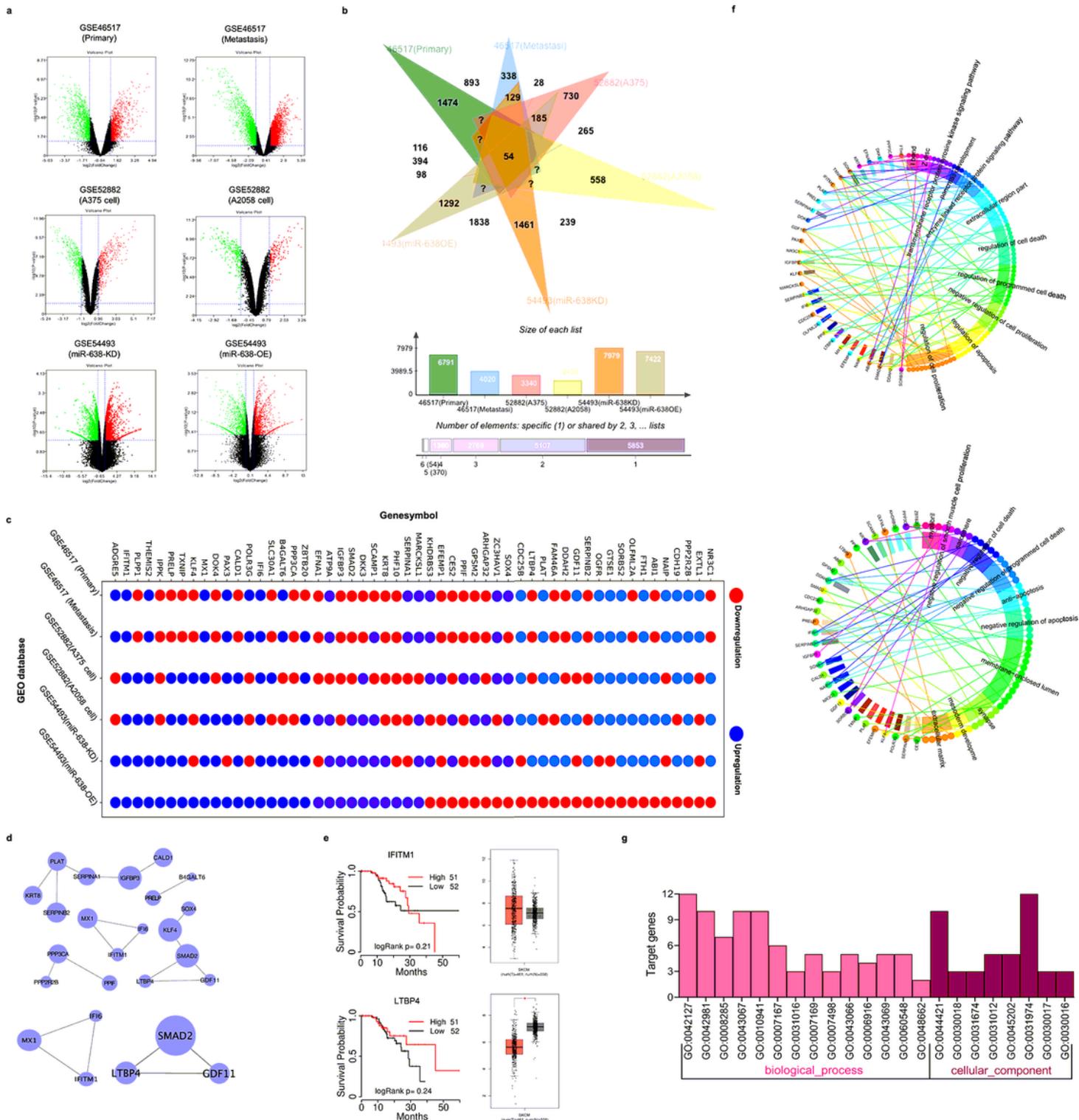


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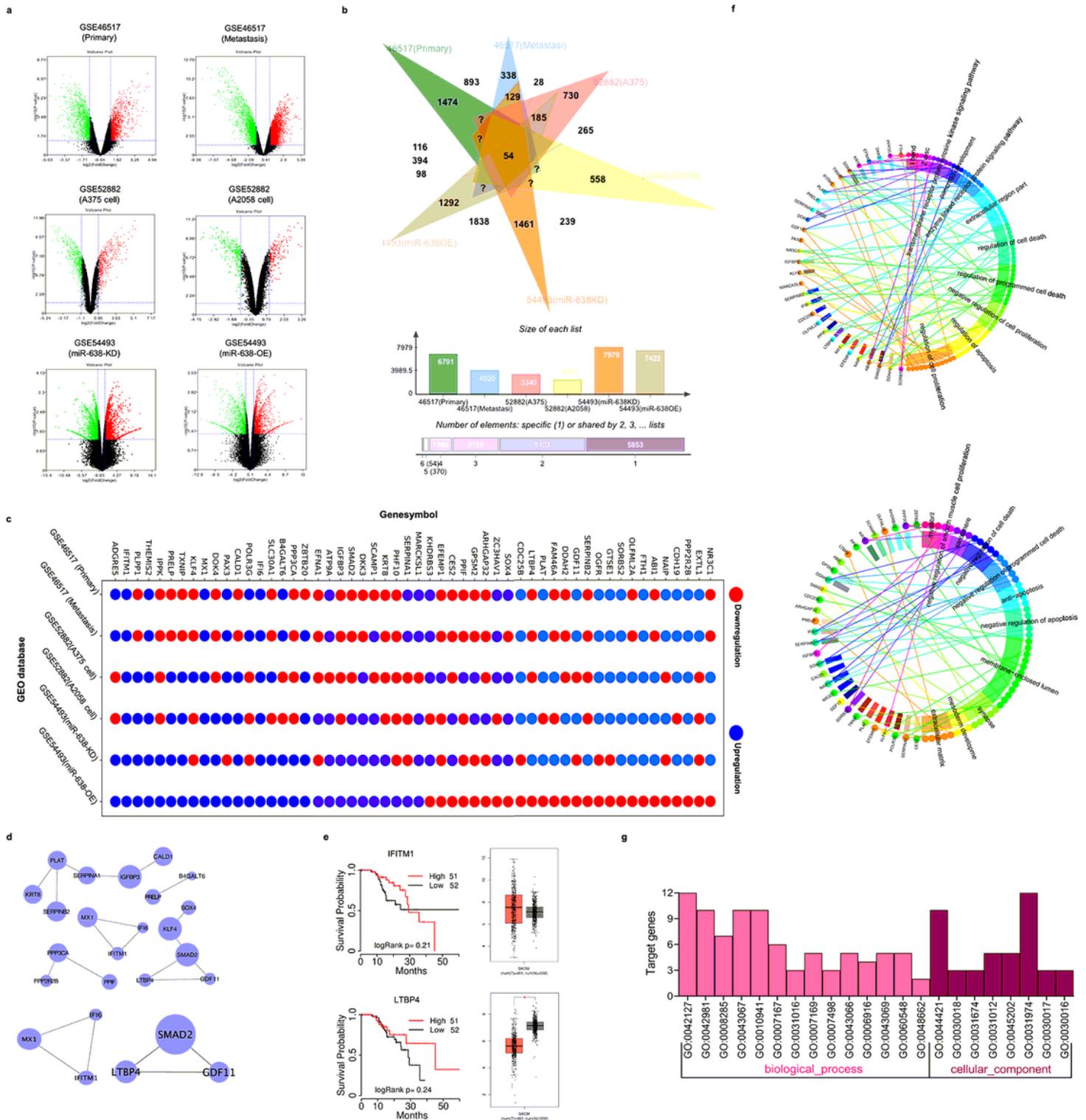


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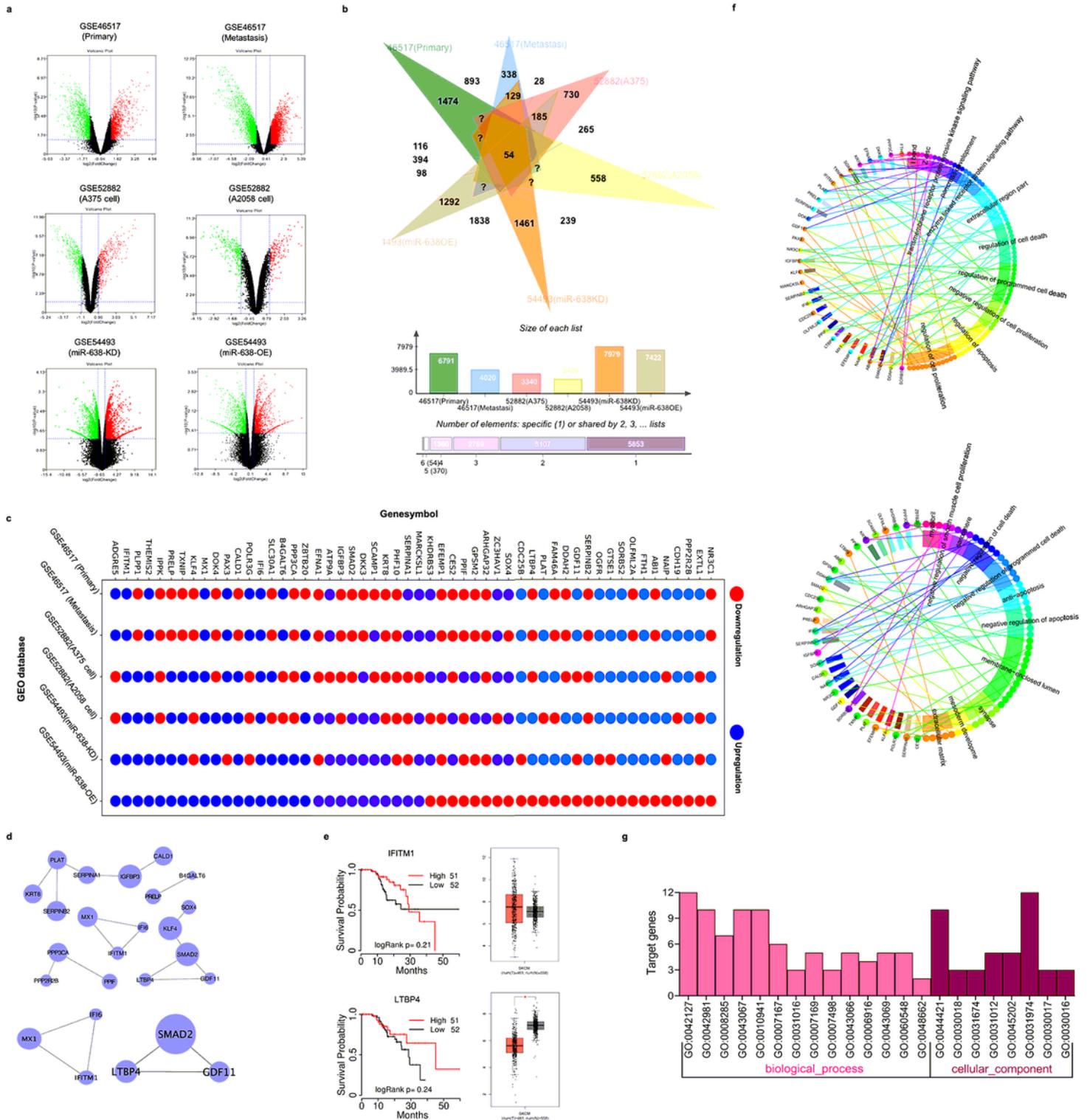


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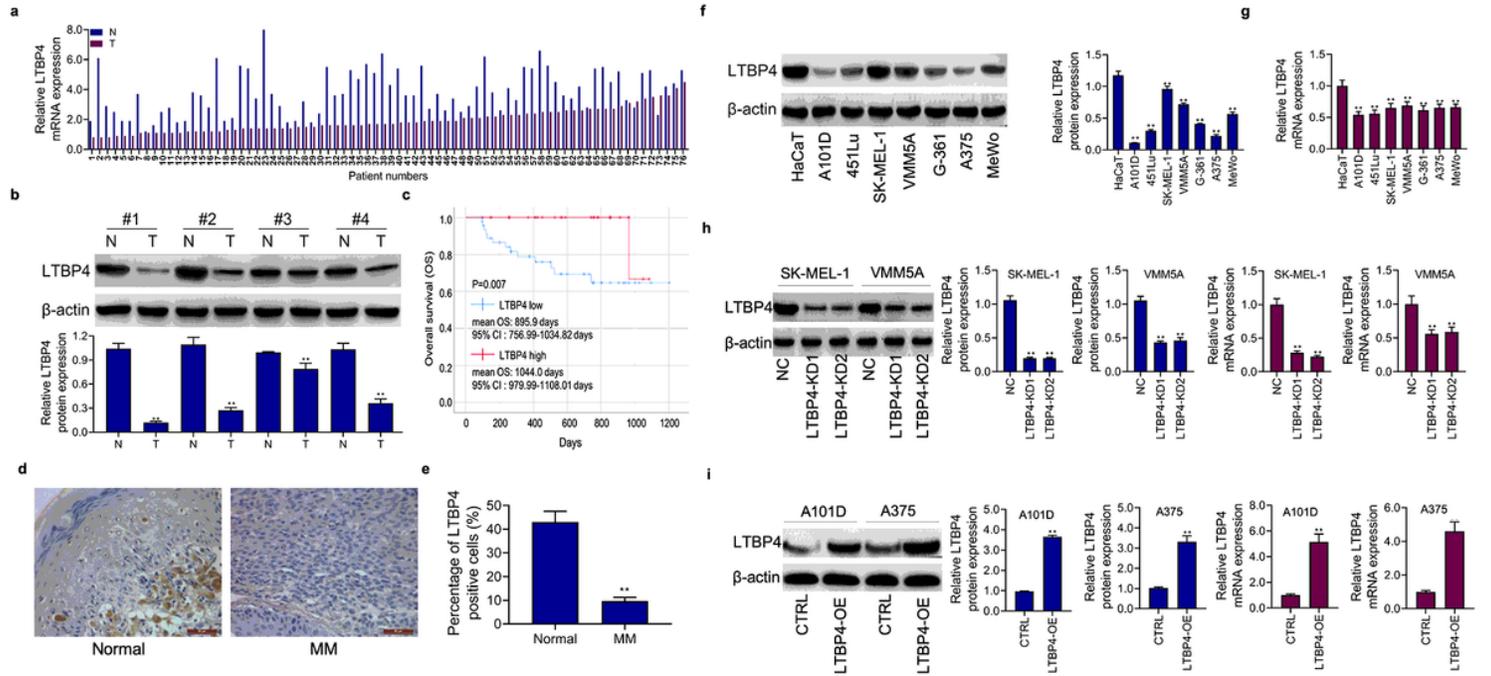


Figure 2

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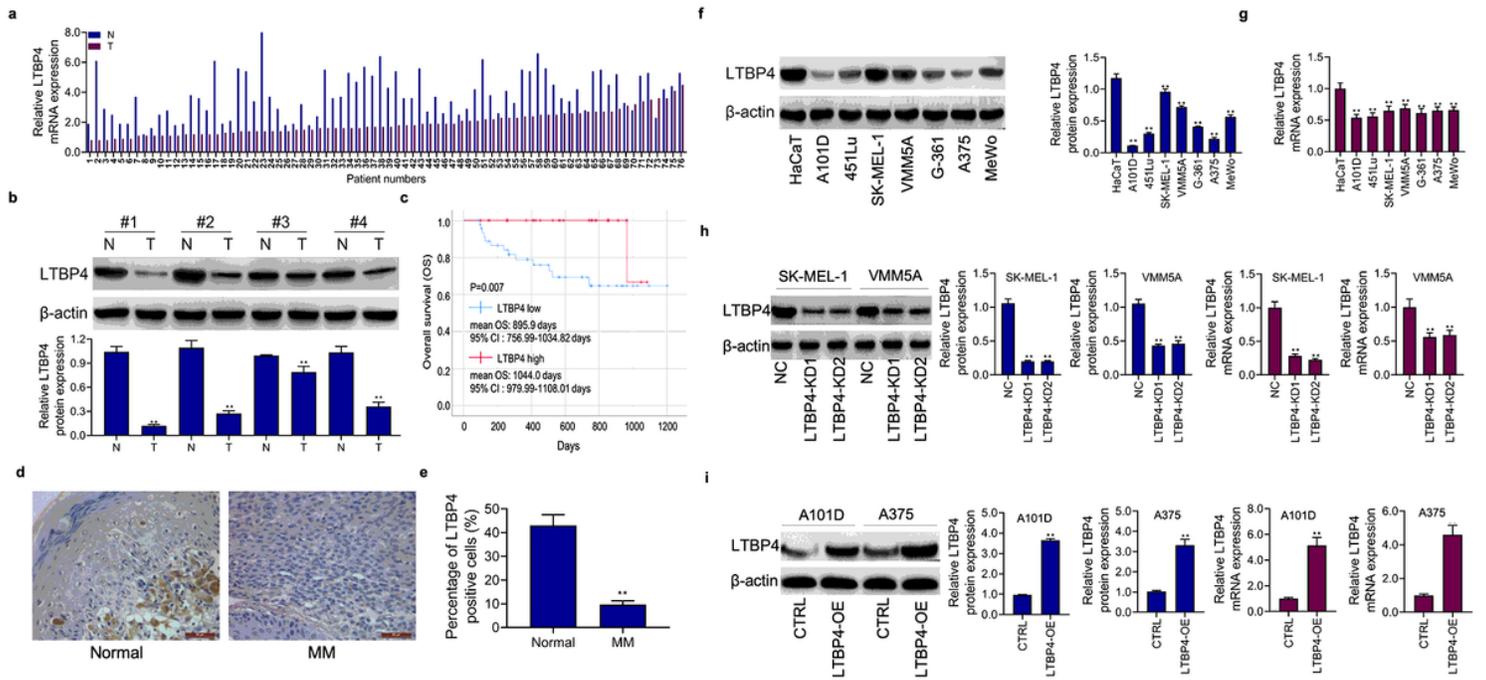


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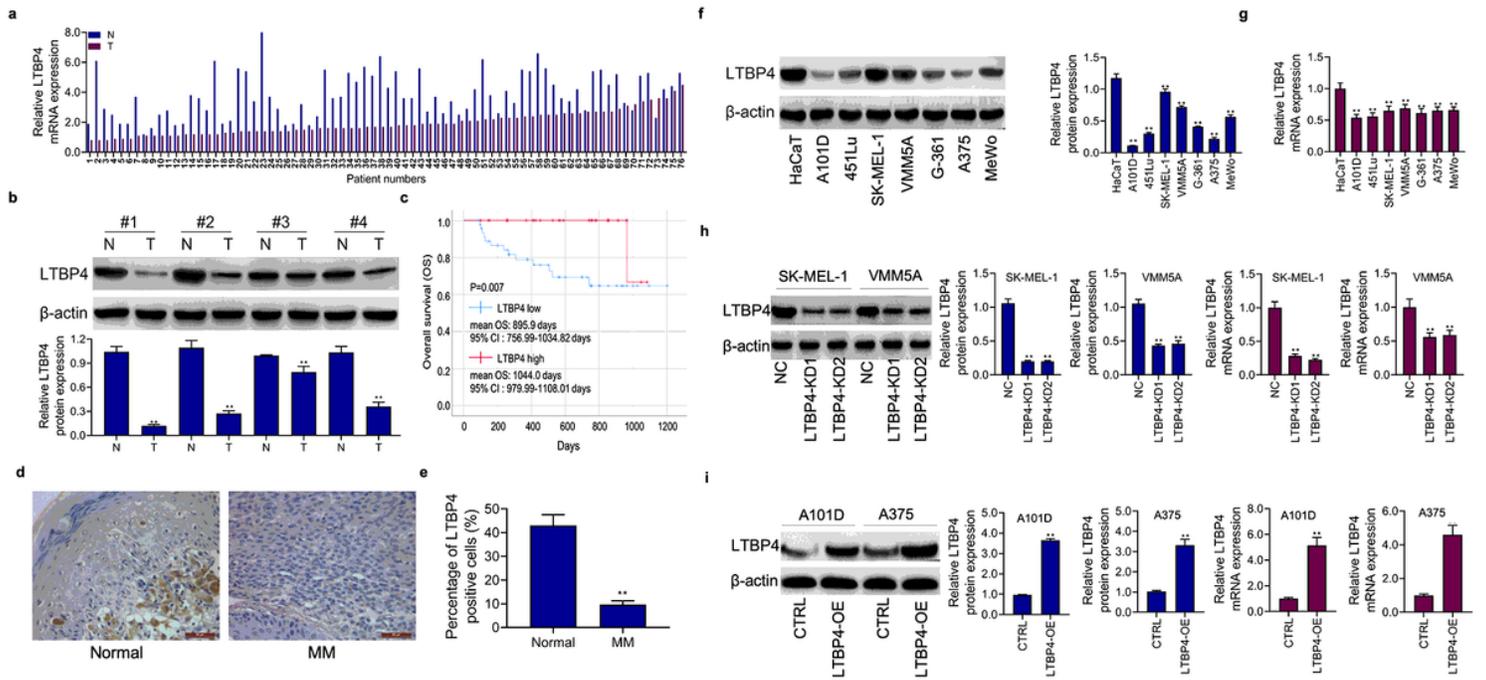


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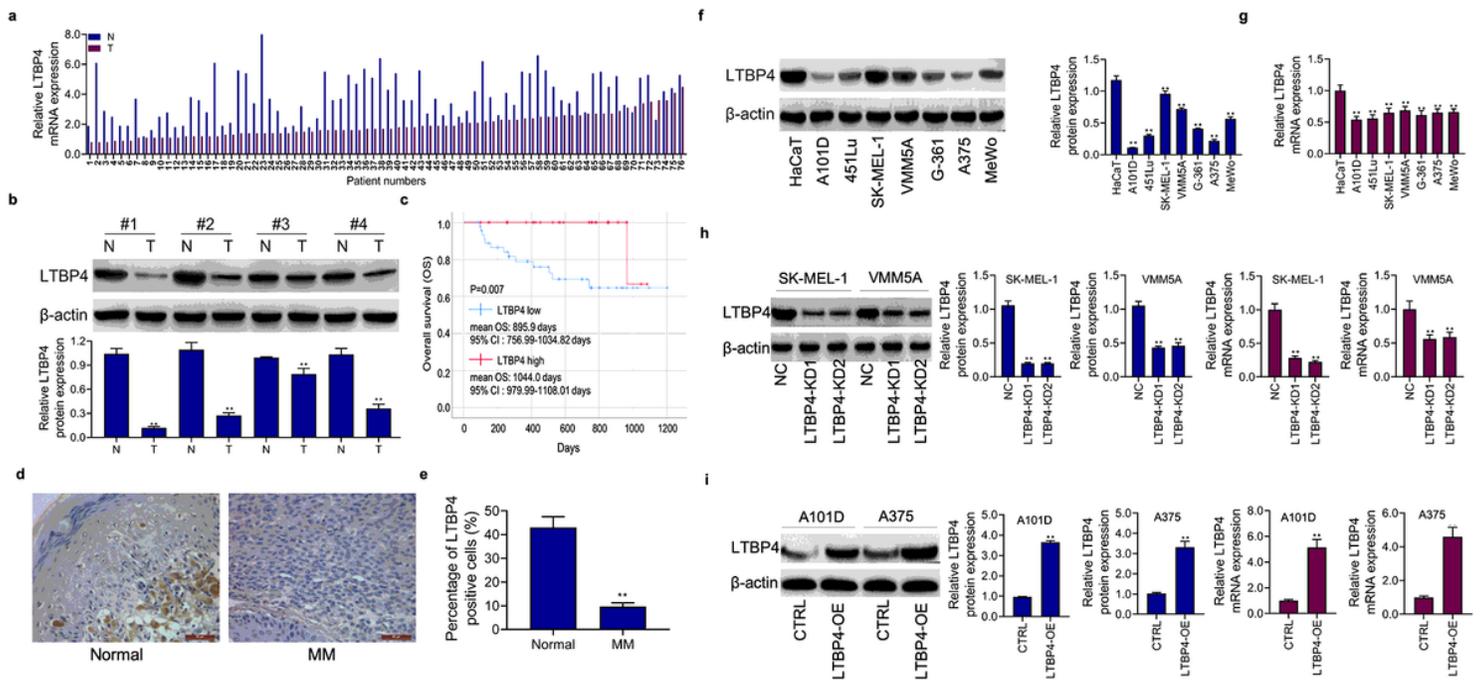


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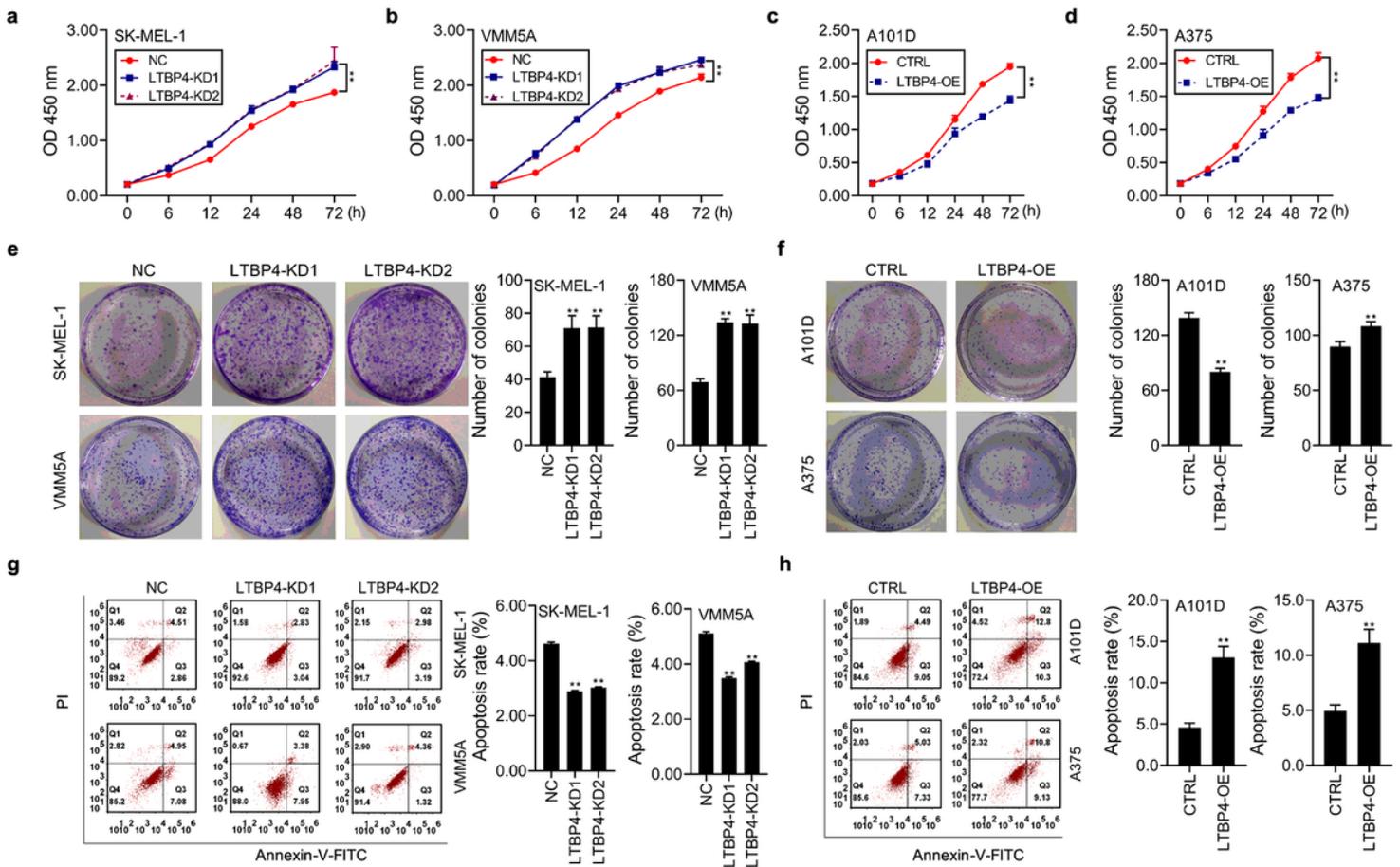


Figure 3

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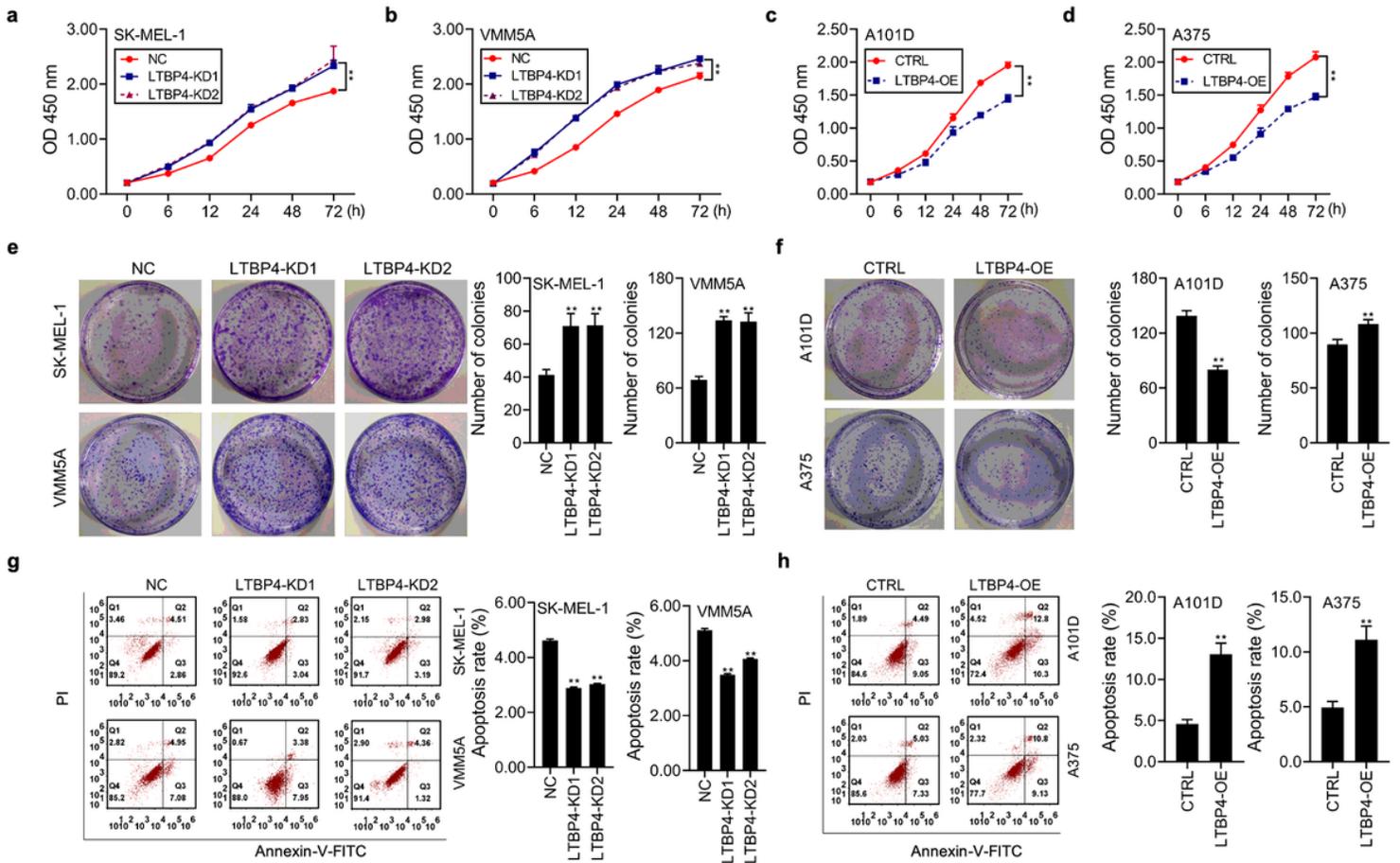


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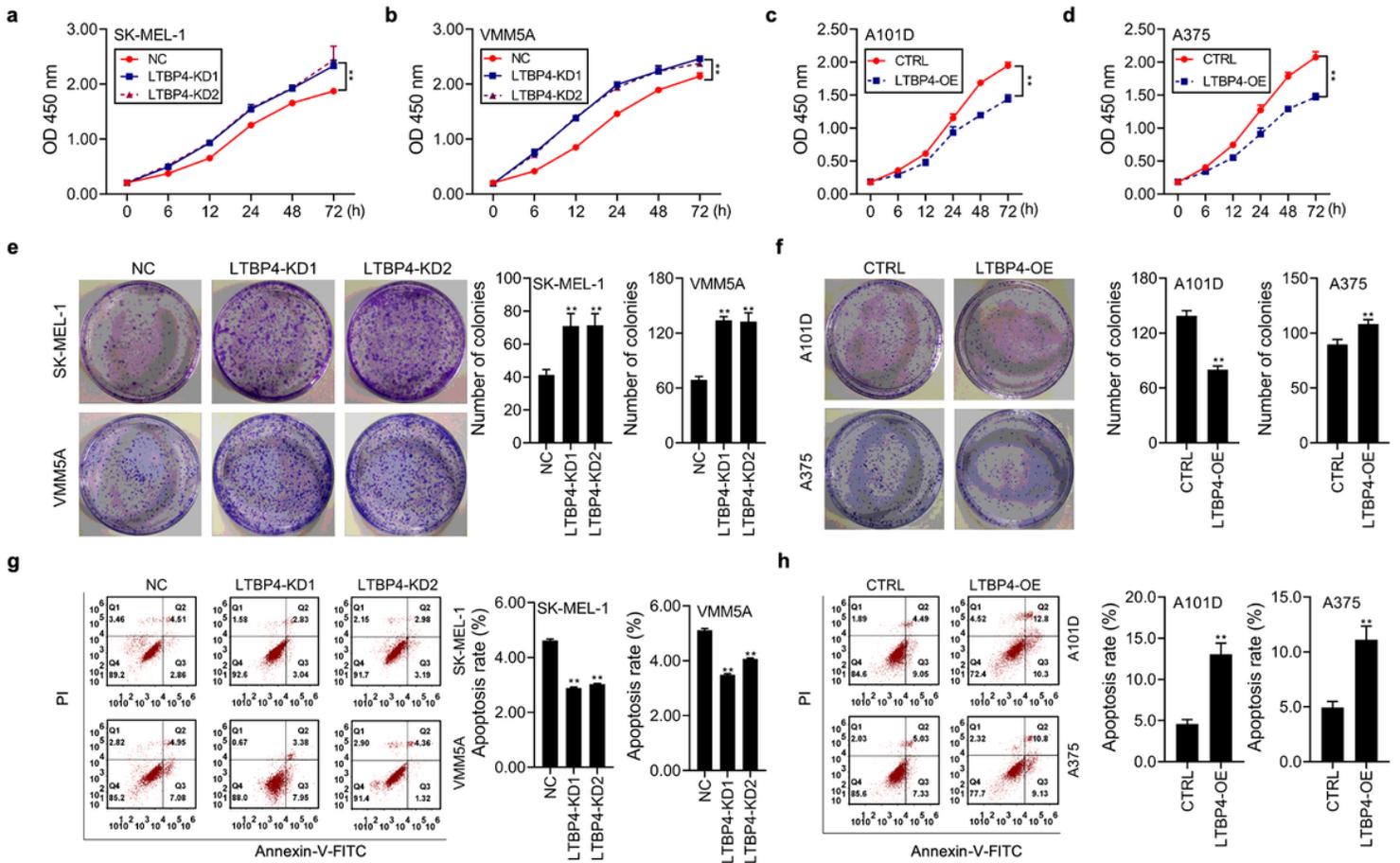


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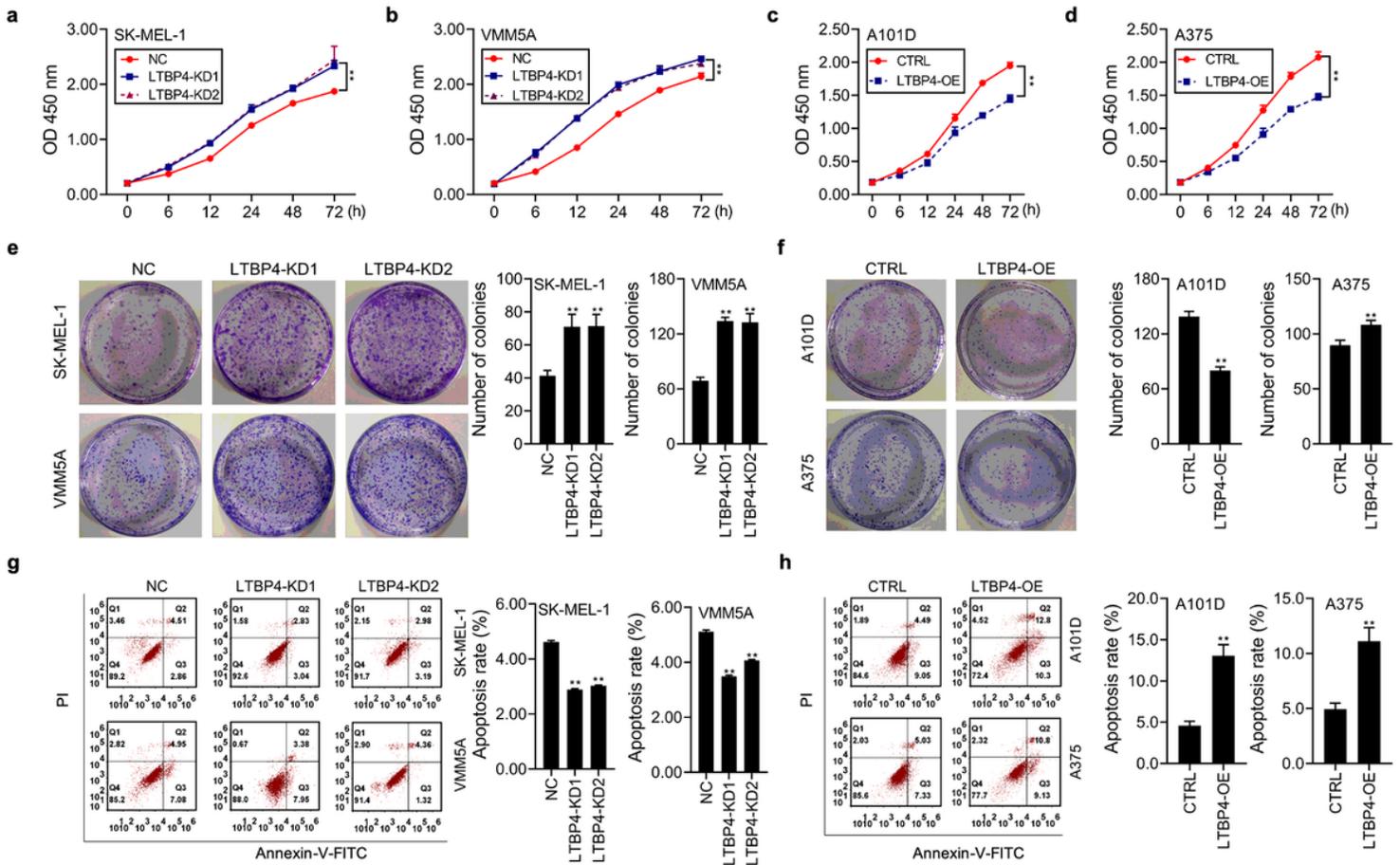


Figure 3

LTBP4 significantly regulated the proliferation and apoptosis in melanoma cell lines. (a and b) The viability of shRNA-LTBP4#1 or #2 transfected SK-MEL-1 and VMM5A cells was detected by CCK-8 assay. (c and d) The viability of LTBP4 expressing plasmids transfected A101D and A375 cells was detected by CCK-8 assay. (e) The proliferation of shRNA-LTBP4#1 or #2 transfected SK-MEL-1 and VMM5A cells was detected by colony formation assay. (f) The proliferation of LTBP4 expressing plasmids transfected A101D and A375 cells was detected by colony formation assay. (g) The apoptosis level of shRNA-LTBP4#1 or #2 transfected SK-MEL-1 and VMM5A cells was detected by flow cytometry assay. (h) The apoptosis level of LTBP4 expressing plasmids transfected A101D and A375 cells by flow cytometry assay. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. NC/CTRL group.

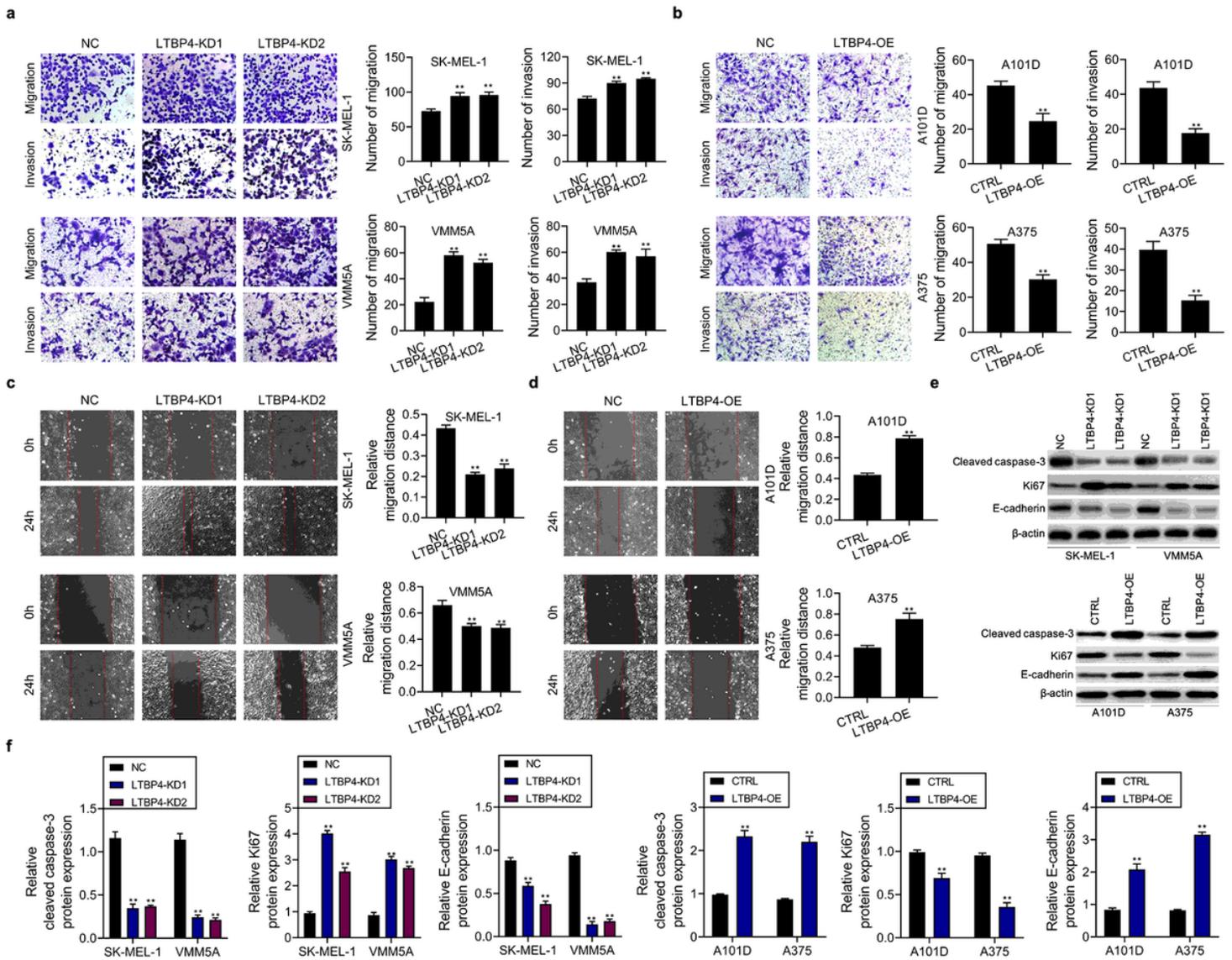


Figure 4

LTBP4 significantly regulated the invasion and migration in melanoma cell lines, and closely related to the expressions of cleaved caspase-3, Ki67 and E-cadherin. (a) The migration and invasion of shRNA-LTBP4#1 or #2 transfected SK-MEL-1 and VMM5A cells was detected by transwell assay. (b) The migration and invasion of LTBP4 expressing plasmids transfected A101D and A375 cells was detected by transwell assay. (c) The migration of shRNA-LTBP4#1 or #2 transfected SK-MEL-1 and VMM5A cells was detected by transwell assay. (d) The migration of LTBP4 expressing plasmids transfected A101D and A375 cells was detected by wound healing assay. (e and f) The protein levels of cleaved caspase-3, Ki67, and E-cadherin in shRNA-LTBP4#1 or #2 transfected SK-MEL-1 and VMM5A cells or LTBP4 expressing plasmids transfected A101D and A375 cells were detected by western blotting assay. β -actin was used as a load control. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. NC/CTRL group.

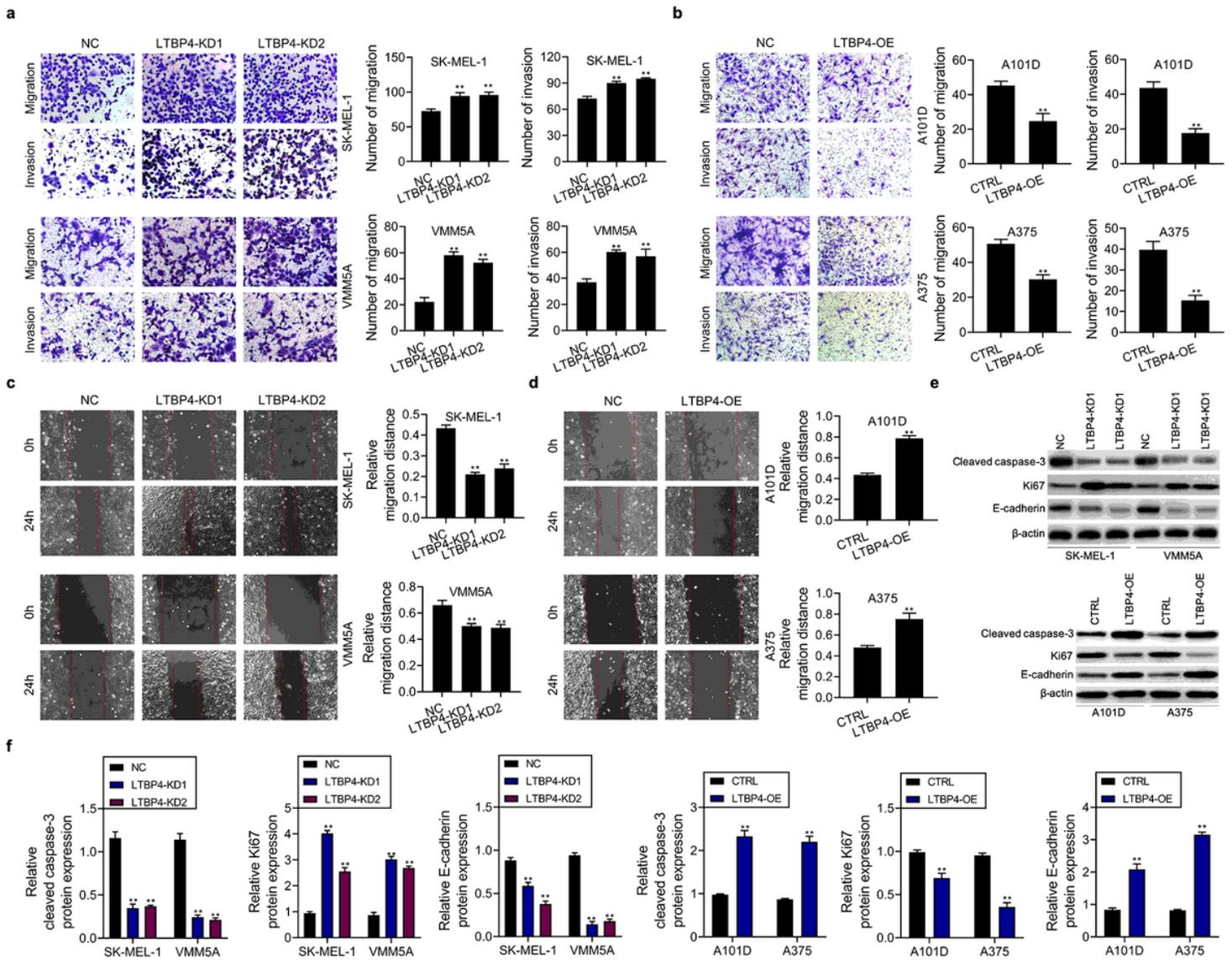


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LTBP4 significantly regulated the invasion and migration in melanoma cell lines, and closely related to the expressions of cleaved caspase-3, Ki67 and E-cadherin. (a) The migration and invasion of shRNA-LTBP4#1 or #2 transfected SK-MEL-1 and VMM5A cells was detected by transwell assay. (b) The migration and invasion of LTBP4 expressing plasmids transfected A101D and A375 cells was detected by transwell assay. (c) The migration of shRNA-LTBP4#1 or #2 transfected SK-MEL-1 and VMM5A cells was detected by transwell assay. (d) The migration of LTBP4 expressing plasmids transfected A101D and A375 cells was detected by wound healing assay. (e and f) The protein levels of cleaved caspase-3, Ki67, and E-cadherin in shRNA-LTBP4#1 or #2 transfected SK-MEL-1 and VMM5A cells or LTBP4 expressing plasmids transfected A101D and A375 cells were detected by western blotting assay. β -actin was used as a load control. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. NC/CTRL group.

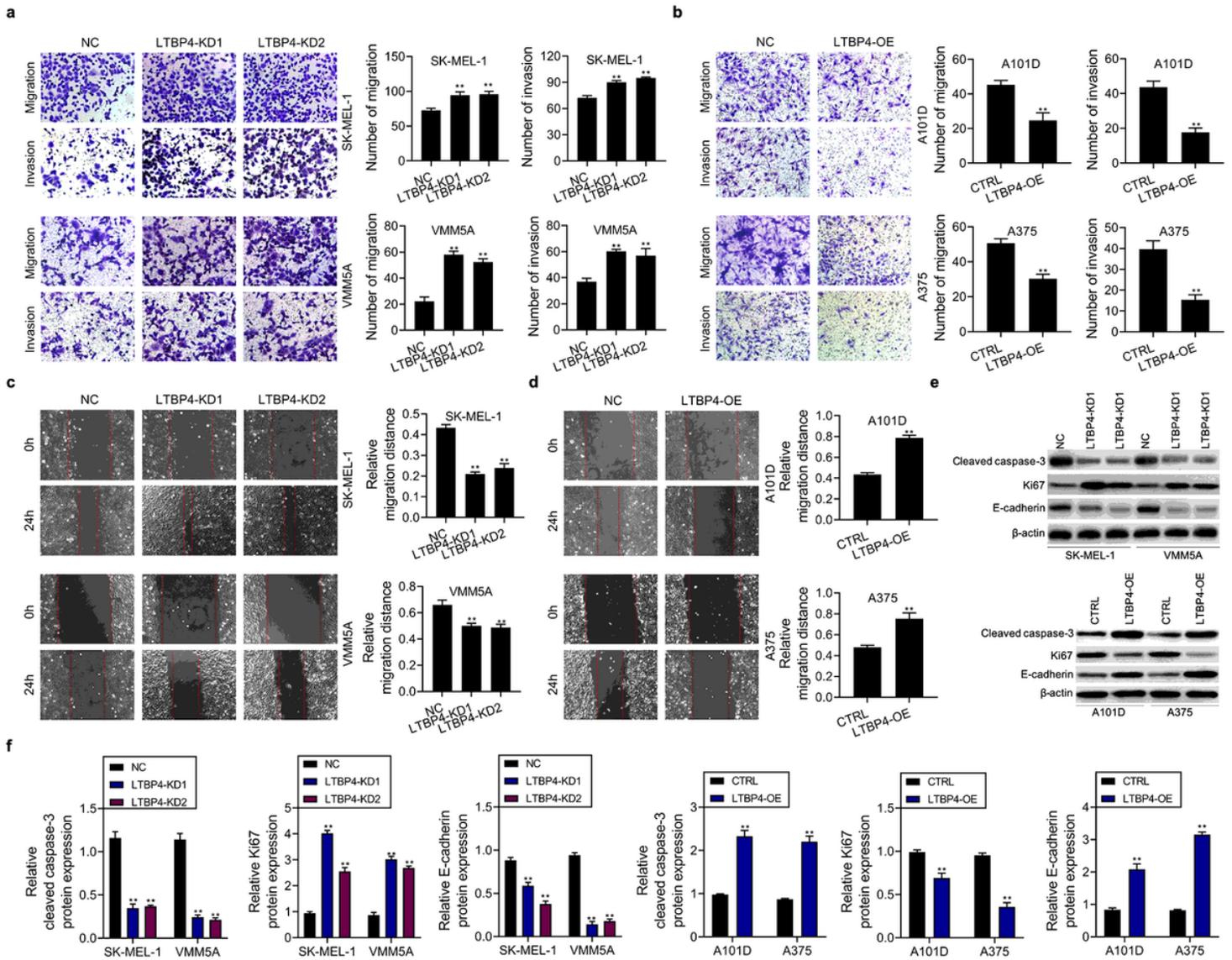


Figure 4

LTBP4 significantly regulated the invasion and migration in melanoma cell lines, and closely related to the expressions of cleaved caspase-3, Ki67 and E-cadherin. (a) The migration and invasion of shRNA-LTBP4#1 or #2 transfected SK-MEL-1 and VMM5A cells was detected by transwell assay. (b) The migration and invasion of LTBP4 expressing plasmids transfected A101D and A375 cells was detected by transwell assay. (c) The migration of shRNA-LTBP4#1 or #2 transfected SK-MEL-1 and VMM5A cells was detected by transwell assay. (d) The migration of LTBP4 expressing plasmids transfected A101D and A375 cells was detected by wound healing assay. (e and f) The protein levels of cleaved caspase-3, Ki67, and E-cadherin in shRNA-LTBP4#1 or #2 transfected SK-MEL-1 and VMM5A cells or LTBP4 expressing plasmids transfected A101D and A375 cells were detected by western blotting assay. β -actin was used as a load control. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. NC/CTRL group.

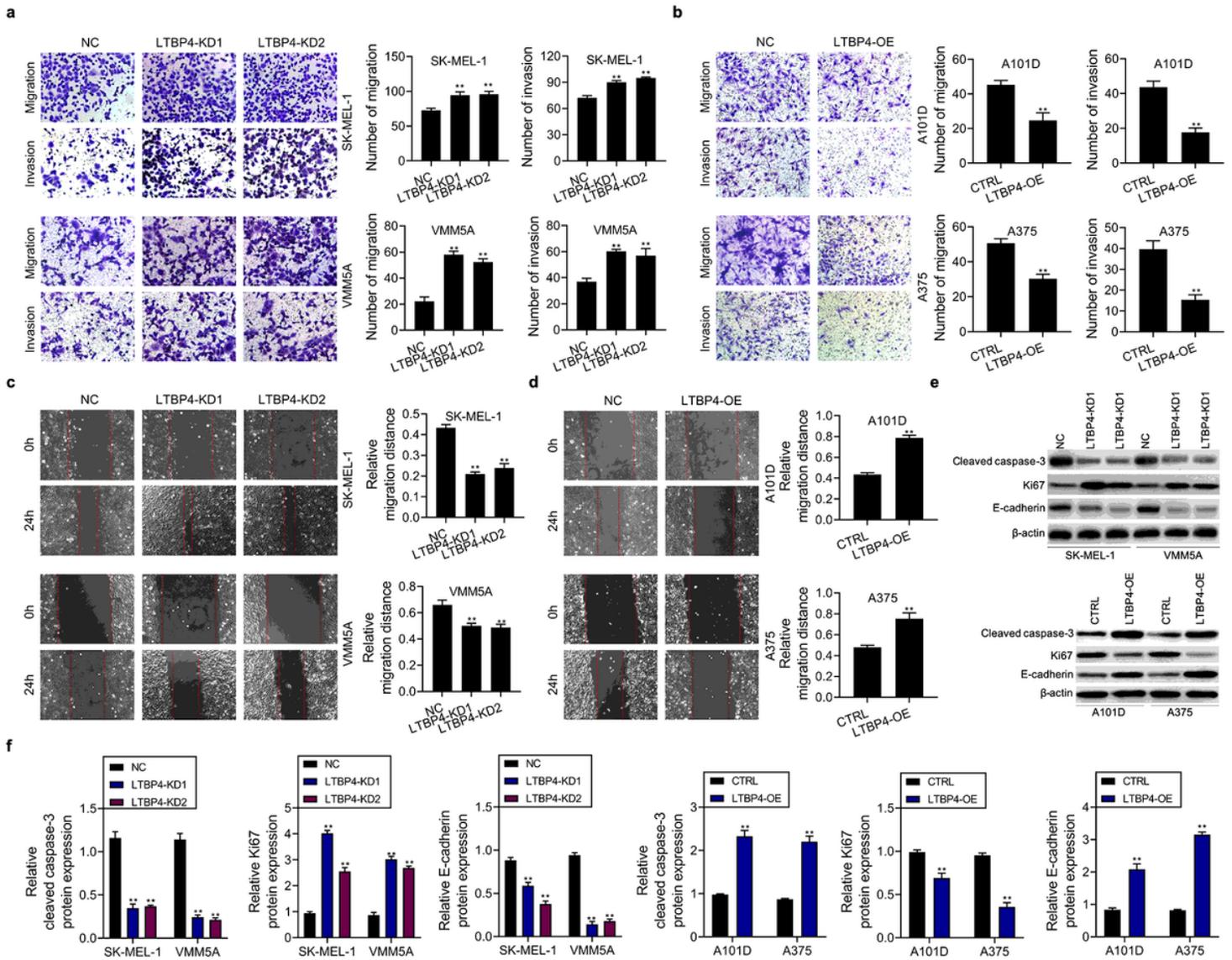


Figure 4

LTBP4 significantly regulated the invasion and migration in melanoma cell lines, and closely related to the expressions of cleaved caspase-3, Ki67 and E-cadherin. (a) The migration and invasion of shRNA-LTBP4#1 or #2 transfected SK-MEL-1 and VMM5A cells was detected by transwell assay. (b) The migration and invasion of LTBP4 expressing plasmids transfected A101D and A375 cells was detected by transwell assay. (c) The migration of shRNA-LTBP4#1 or #2 transfected SK-MEL-1 and VMM5A cells was detected by transwell assay. (d) The migration of LTBP4 expressing plasmids transfected A101D and A375 cells was detected by wound healing assay. (e and f) The protein levels of cleaved caspase-3, Ki67, and E-cadherin in shRNA-LTBP4#1 or #2 transfected SK-MEL-1 and VMM5A cells or LTBP4 expressing plasmids transfected A101D and A375 cells were detected by western blotting assay. β -actin was used as a load control. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. NC/CTRL group.

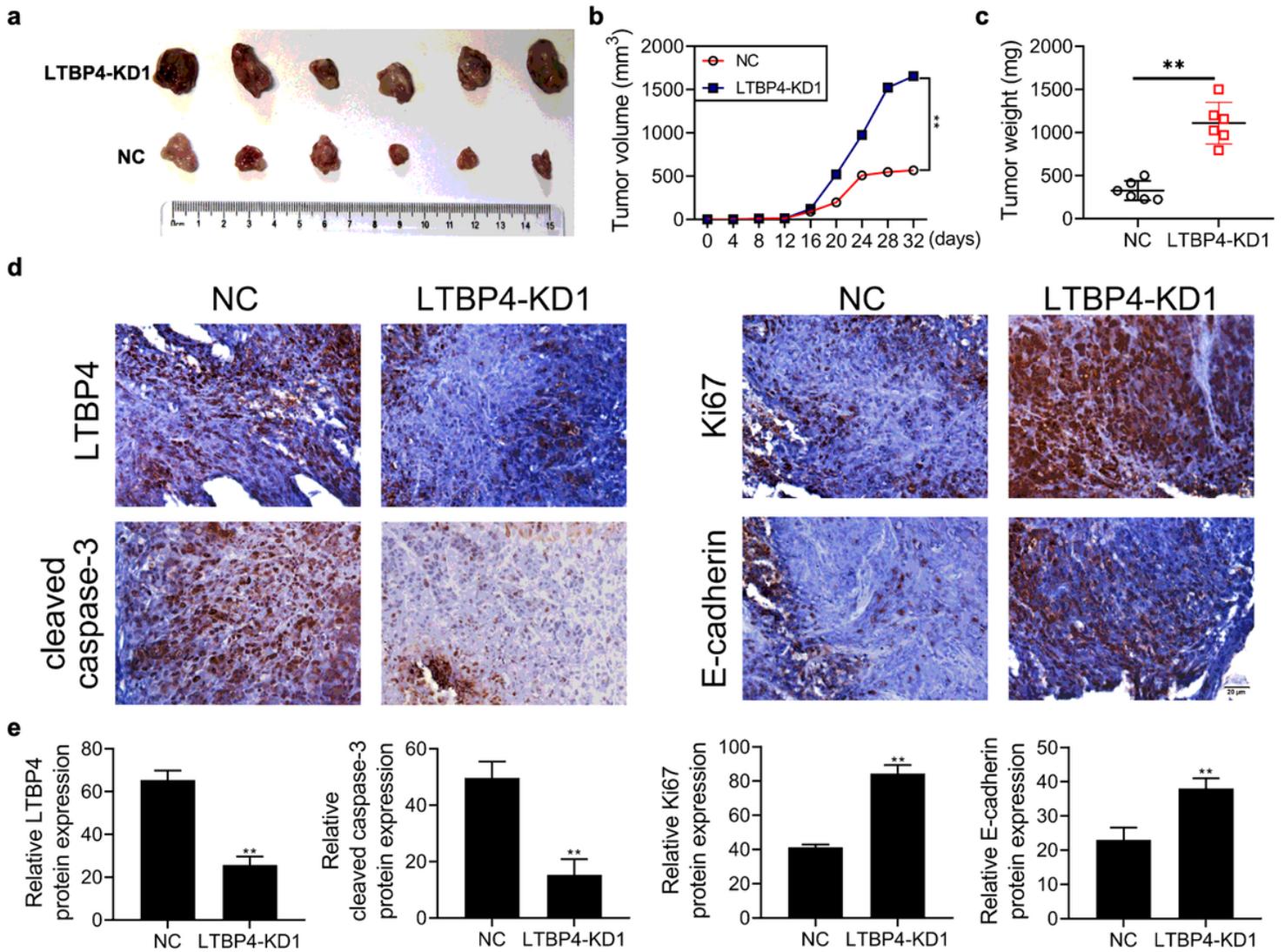


Figure 5

Silencing of LTBP4 promoted the oncogenicity of melanoma cell in vivo SK-MEL-1 cells transfected with shRNA-LTBP4 or NC were subcutaneously injected into the nude mice, (a) The nude mice were sacrificed, and the tumors were collected after 30 day; (b) the volume of the tumors were determined; (c) the weight of the tumors were determined; (d and e) the expression of Ki67, cleaved caspase-3, Ki67 and E-cadherin in the tumors collected from different groups were determined using IHC staining. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. NC group.

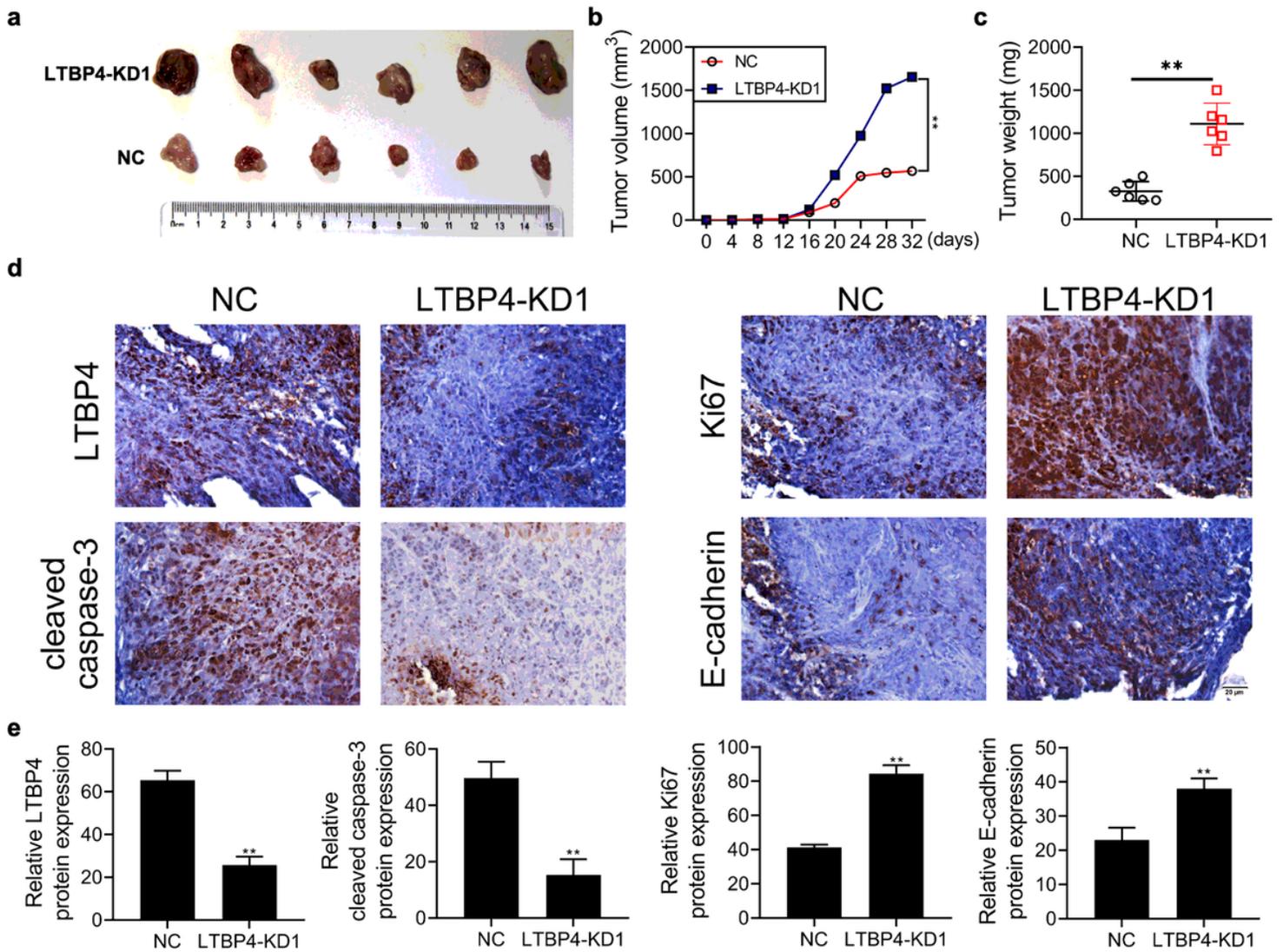


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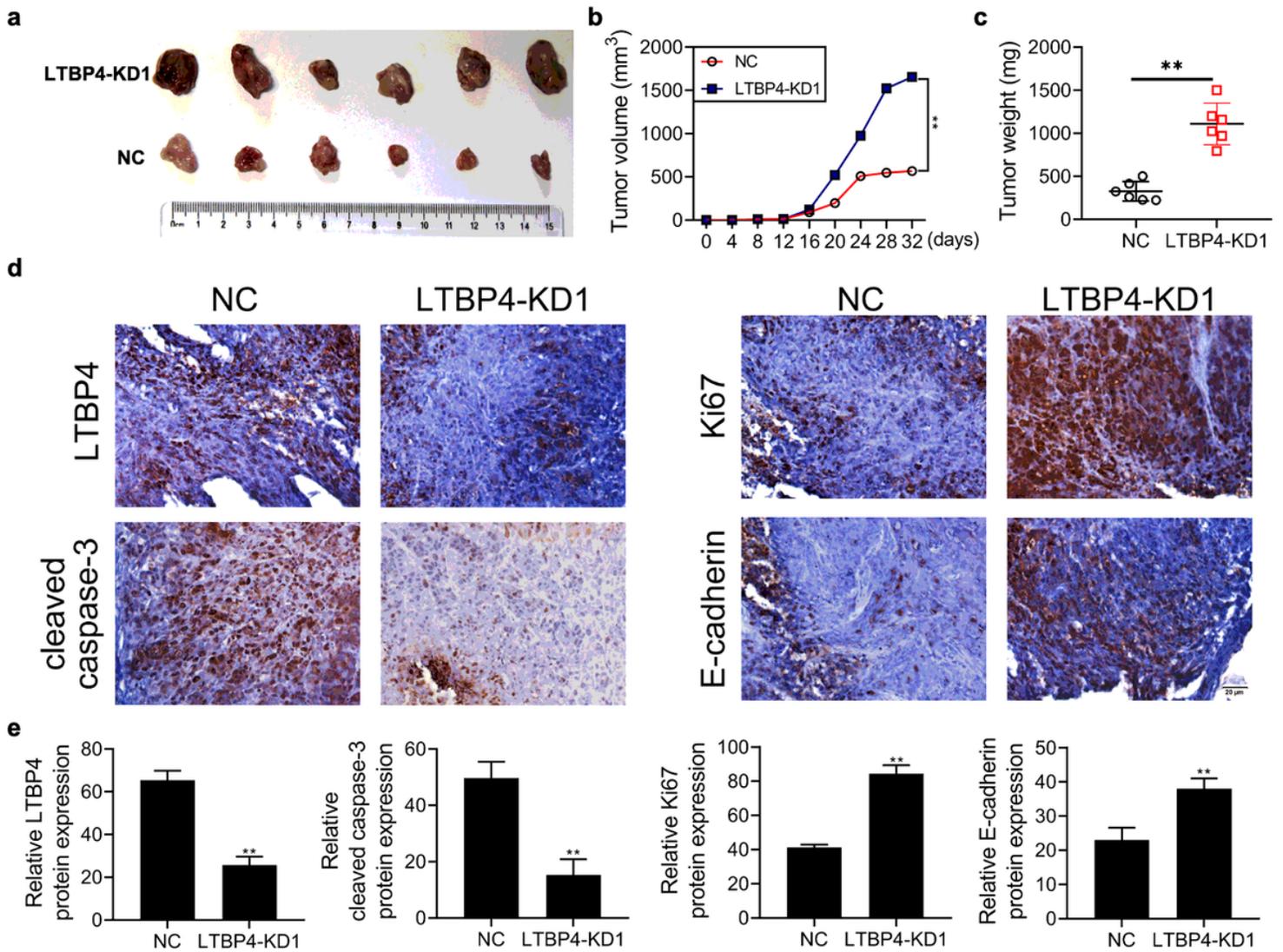


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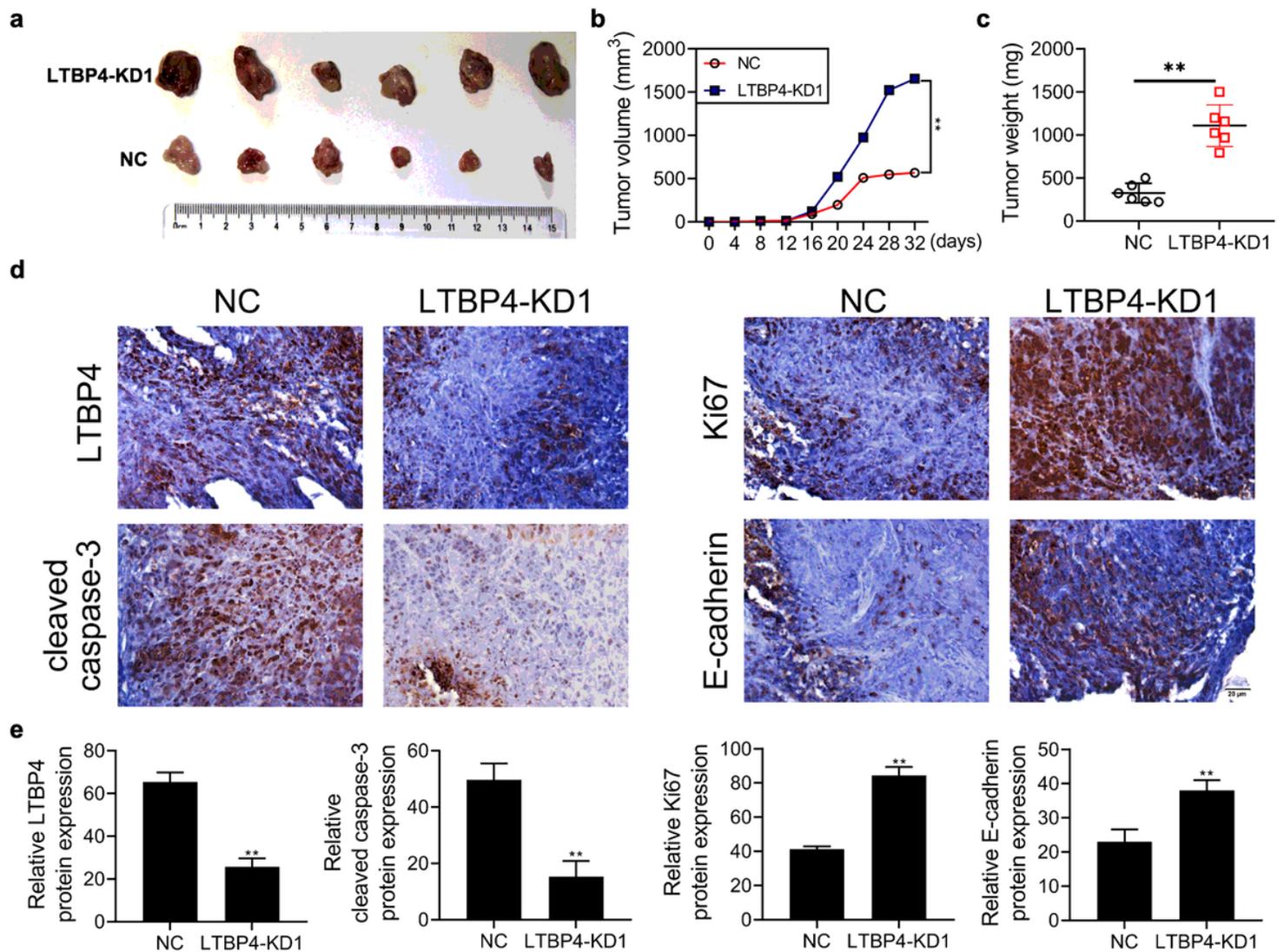


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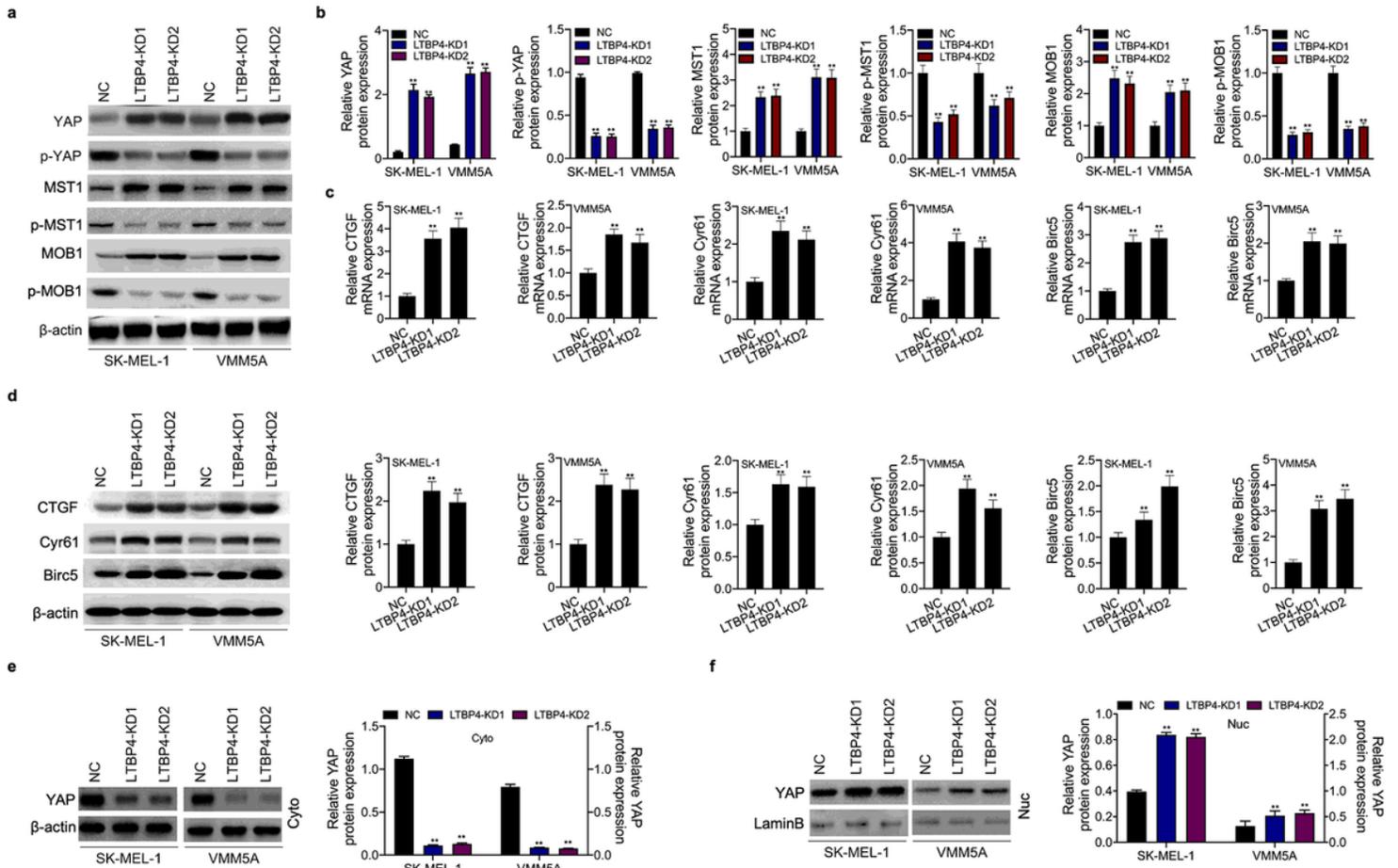


Figure 6

Silencing of LTBP4 suppressed the activation of Hippo signaling pathway via inhibiting the phosphorylation of YAP to promote the nuclear translocation of YAP, thereby promoting the expressions of CTGF, Cyr61 and Birc5 in SK-MEL-1 and VMM5A cells. After shRNA-LTBP4#1 or #2 was transfected into SK-MEL-1 and VMM5A cells, (a and b) the expressions of YAP, MST1, and MOB1 and the phosphorylation level of YAP, MST1, and MOB1 were detected by western blotting assay, (c) the mRNA levels of CTGF, Cyr61, and Birc5 were detected by RT-PCR assay, (d) the protein levels of CTGF, Cyr61, and Birc5 were detected by western blotting assay, (e) the protein levels of YAP in cytoplasm by western blotting assay, and (f) the protein levels of YAP in nucleus by western blotting assay. β -actin and LaminB were used as a load control. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. NC group.

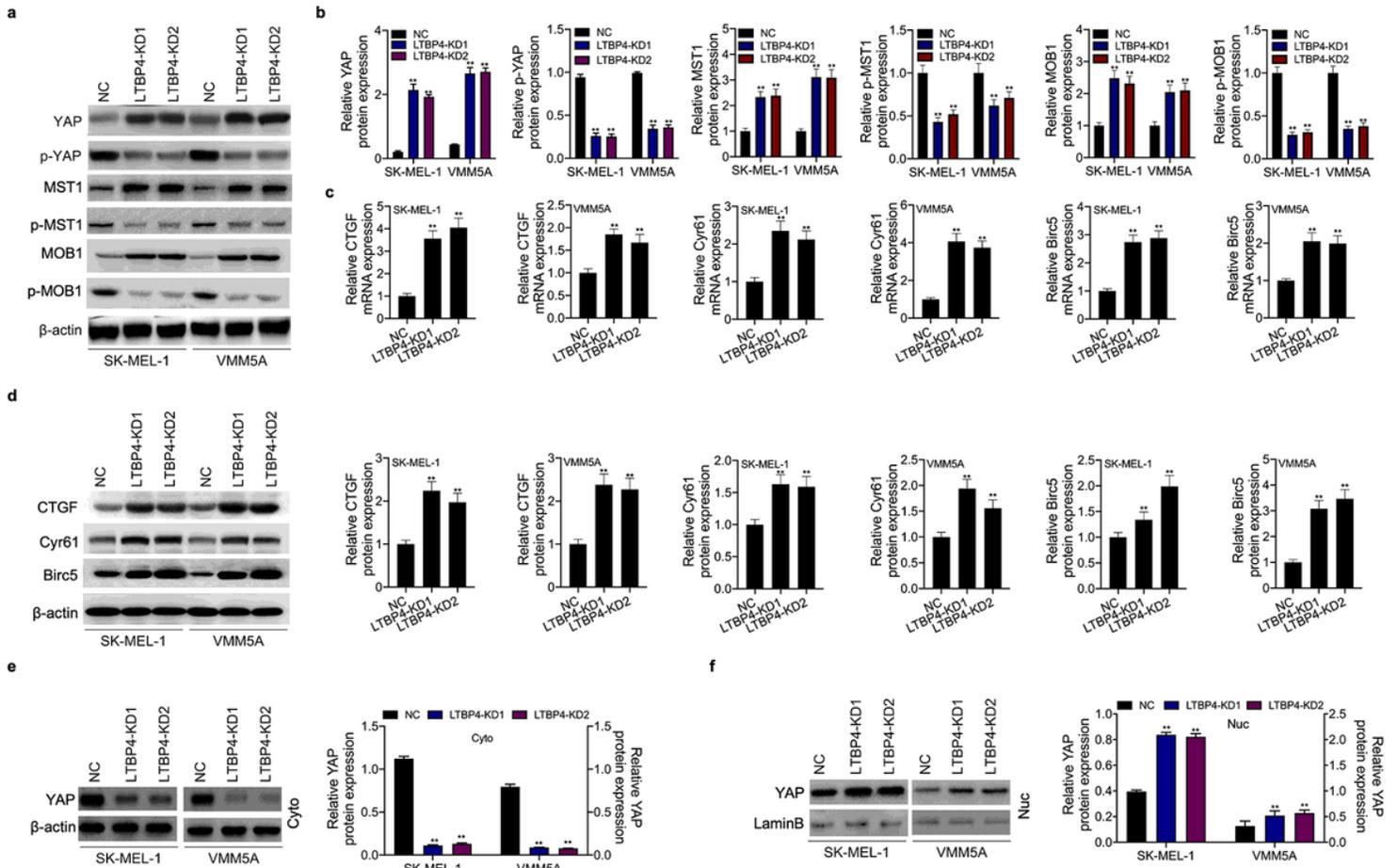


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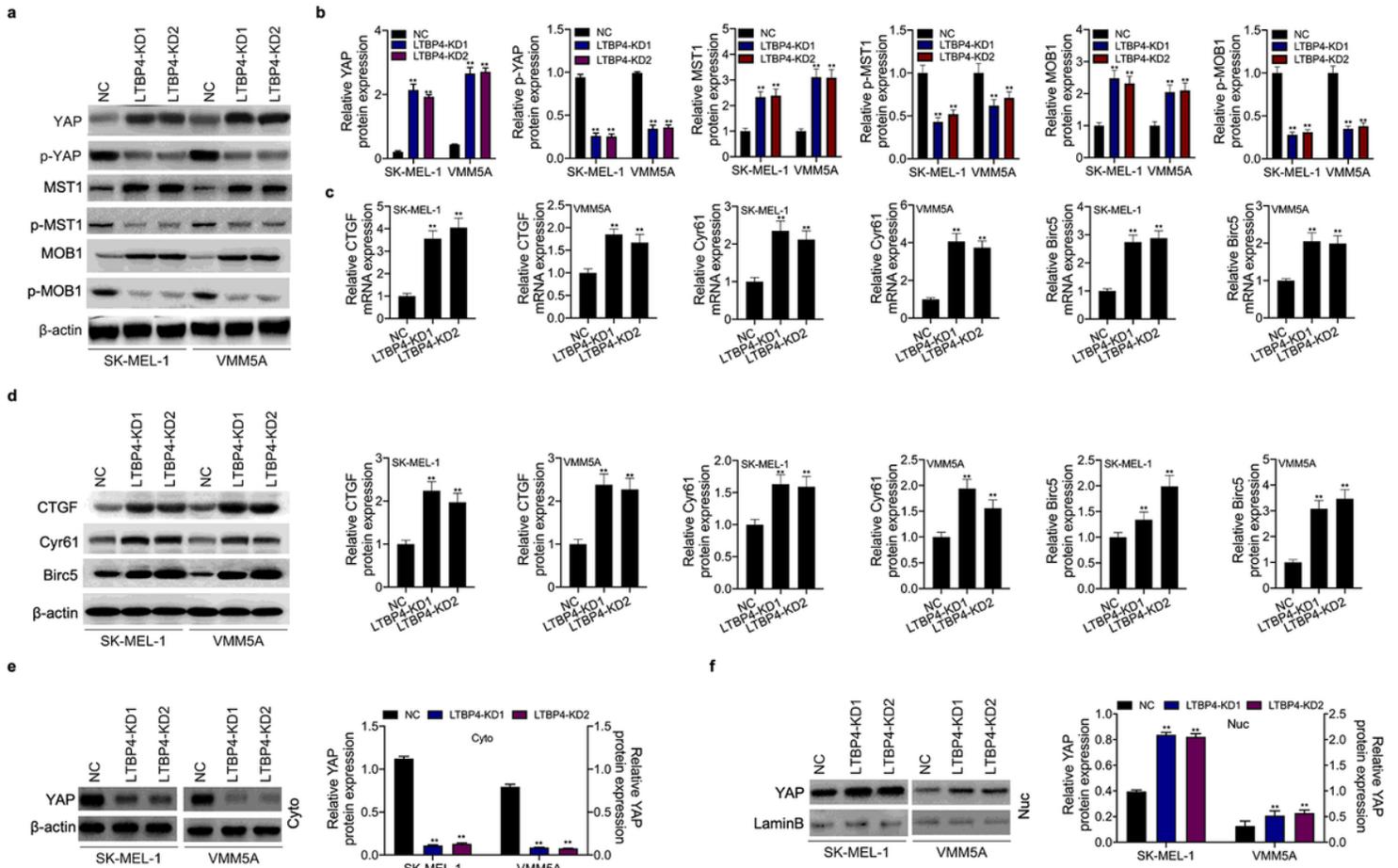


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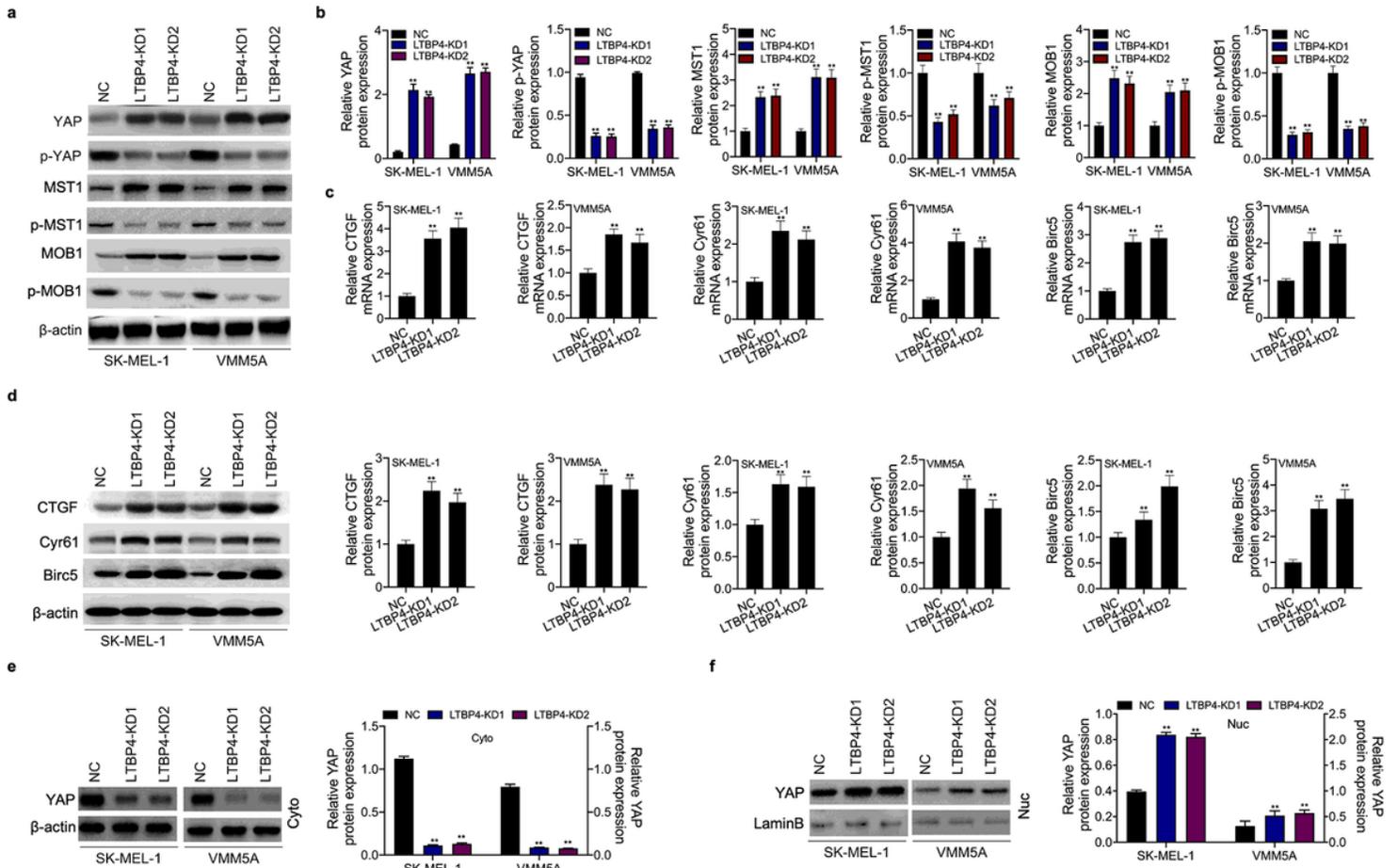


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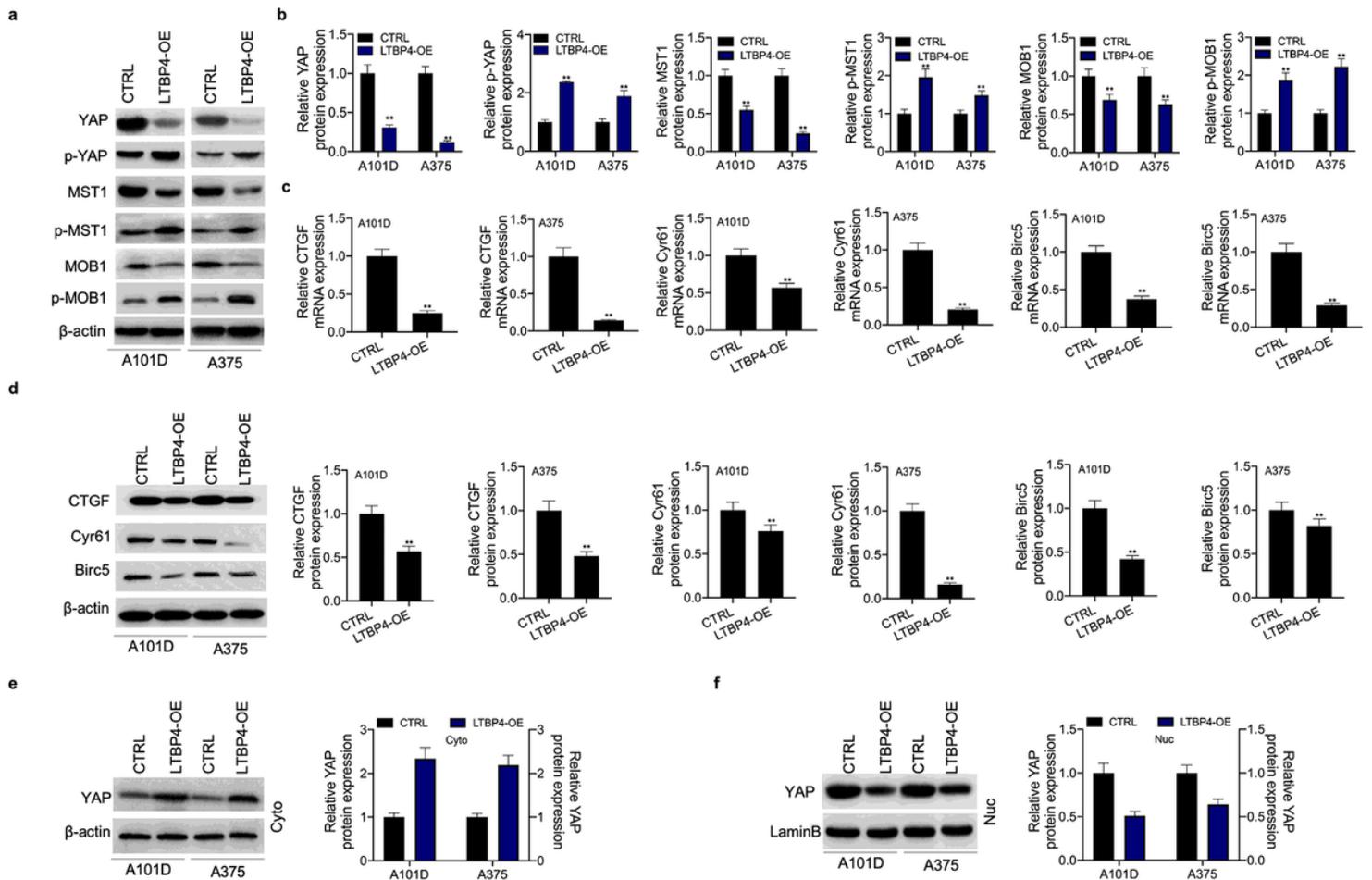


Figure 7

Overexpression of LTBP4 activated Hippo signaling pathway via promoting the phosphorylation of YAP to inhibit the nuclear translocation of YAP, thereby inhibiting the expressions of CTGF, Cyr61 and Birc5 in A101D and A375 cells. After LTBP4 expressing plasmids were transfected into A101D and A375 cells, (a and b) the expressions of YAP, MST1, and MOB1 and the phosphorylation level of YAP, MST1, and MOB1 were detected by western blotting assay, (c) the mRNA levels of CTGF, Cyr61, and Birc5 were detected by RT-PCR assay, (d) the protein levels of CTGF, Cyr61, and Birc5 were detected by western blotting assay, (e) the protein levels of YAP in cytoplasm by western blotting assay, and (f) the protein levels of YAP in nucleus by western blotting assay. β -actin and LaminB were used as a load control. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. CTRL group.

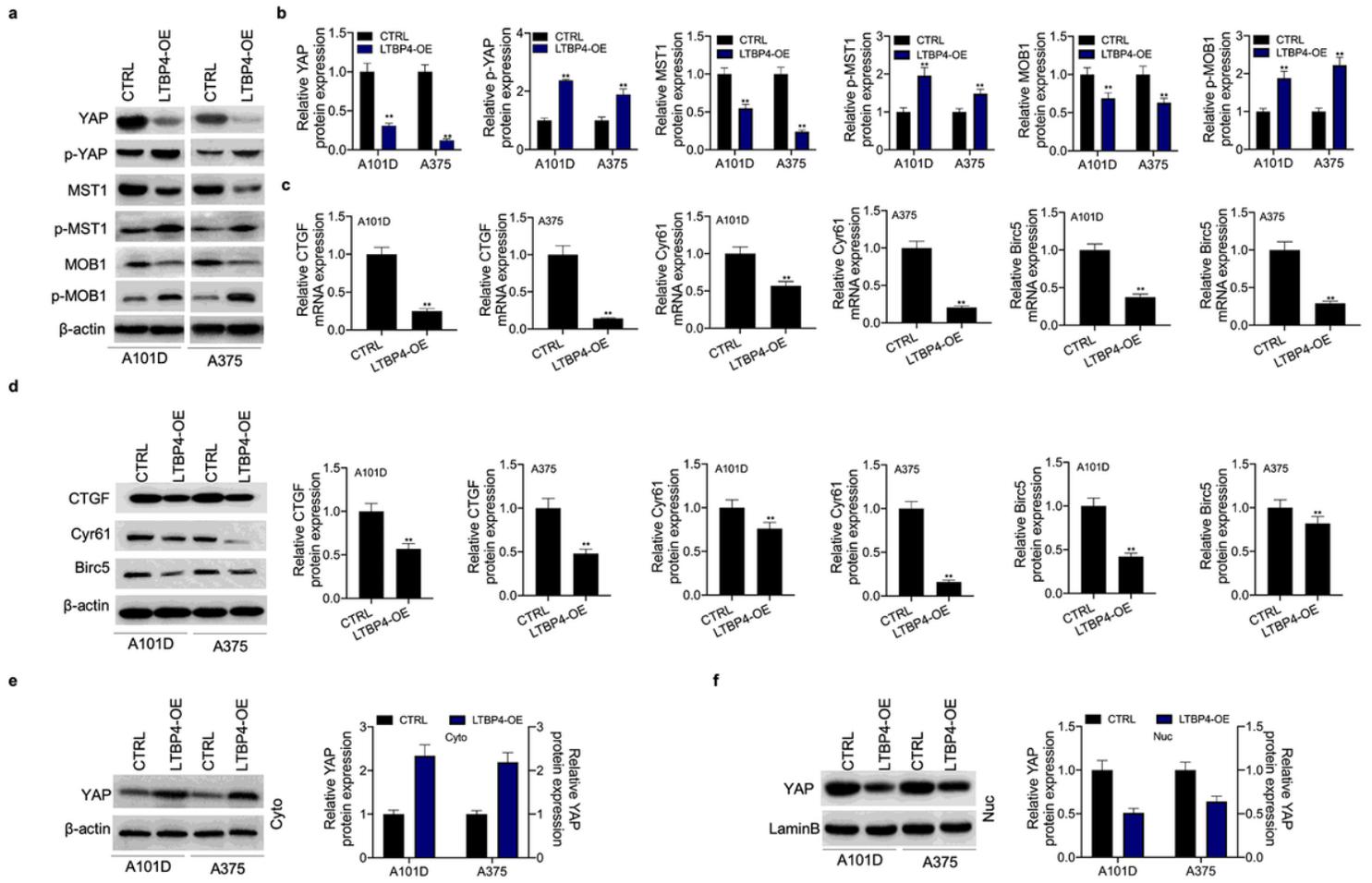


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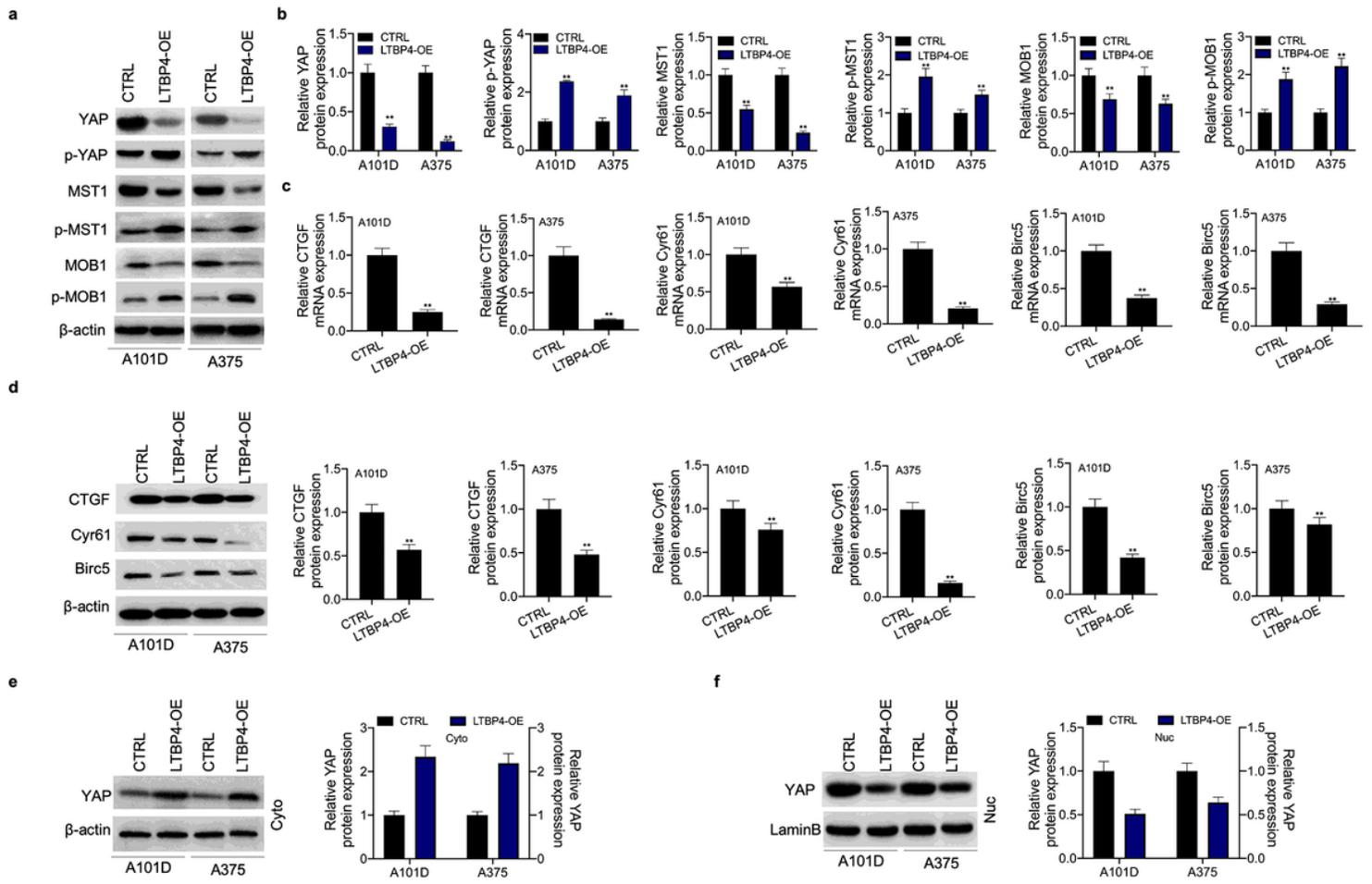


Figure 7

Overexpression of LTBP4 activated Hippo signaling pathway via promoting the phosphorylation of YAP to inhibit the nuclear translocation of YAP, thereby inhibiting the expressions of CTGF, Cyr61 and Birc5 in A101D and A375 cells. After LTBP4 expressing plasmids were transfected into A101D and A375 cells, (a and b) the expressions of YAP, MST1, and MOB1 and the phosphorylation level of YAP, MST1, and MOB1 were detected by western blotting assay, (c) the mRNA levels of CTGF, Cyr61, and Birc5 were detected by RT-PCR assay, (d) the protein levels of CTGF, Cyr61, and Birc5 were detected by western blotting assay, (e) the protein levels of YAP in cytoplasm by western blotting assay, and (f) the protein levels of YAP in nucleus by western blotting assay. β -actin and LaminB were used as a load control. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. CTRL group.

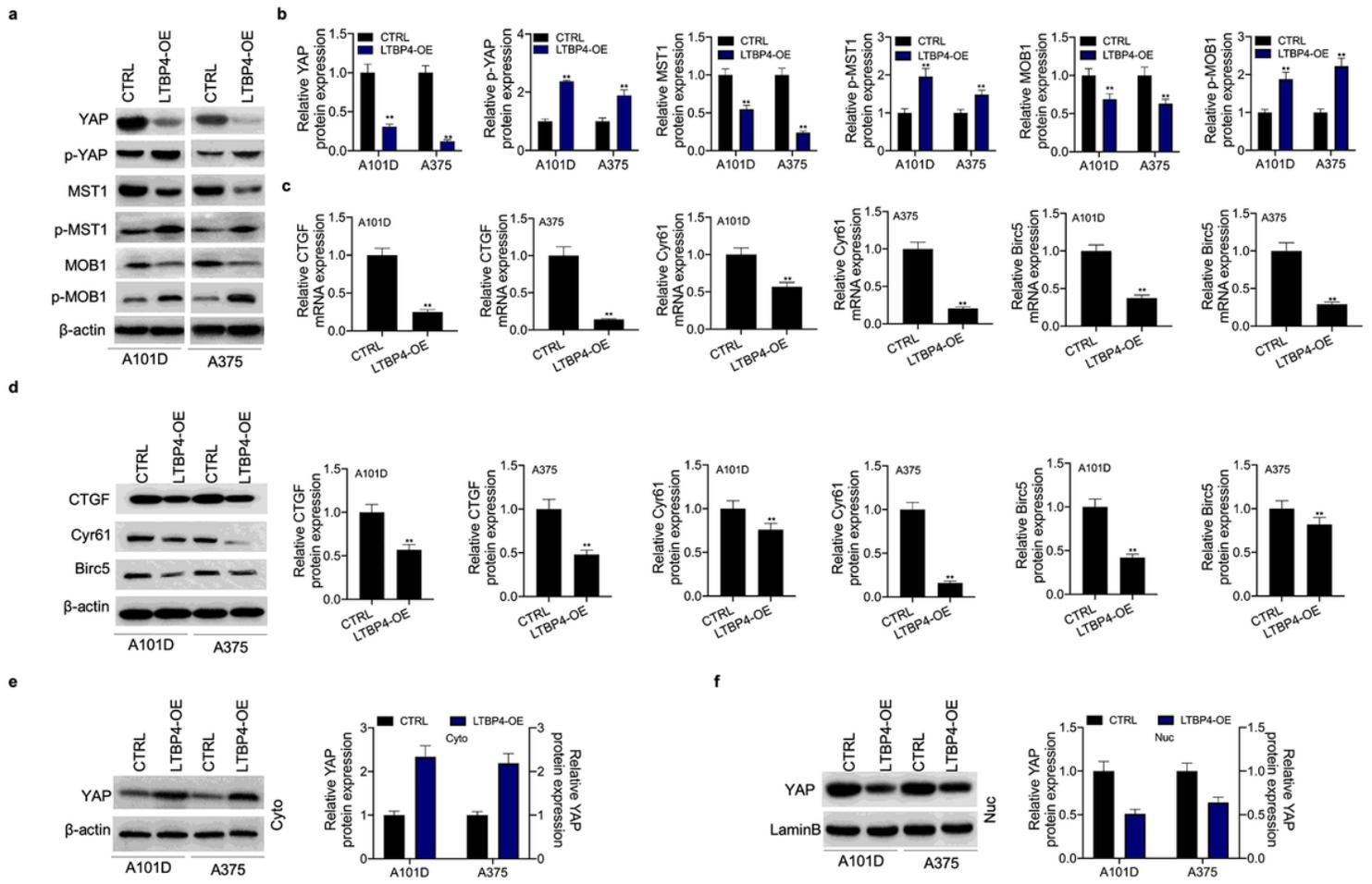


Figure 7

Overexpression of LTBP4 activated Hippo signaling pathway via promoting the phosphorylation of YAP to inhibit the nuclear translocation of YAP, thereby inhibiting the expressions of CTGF, Cyr61 and Birc5 in A101D and A375 cells. After LTBP4 expressing plasmids were transfected into A101D and A375 cells, (a and b) the expressions of YAP, MST1, and MOB1 and the phosphorylation level of YAP, MST1, and MOB1 were detected by western blotting assay, (c) the mRNA levels of CTGF, Cyr61, and Birc5 were detected by RT-PCR assay, (d) the protein levels of CTGF, Cyr61, and Birc5 were detected by western blotting assay, (e) the protein levels of YAP in cytoplasm by western blotting assay, and (f) the protein levels of YAP in nucleus by western blotting assay. β -actin and LaminB were used as a load control. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. CTRL group.

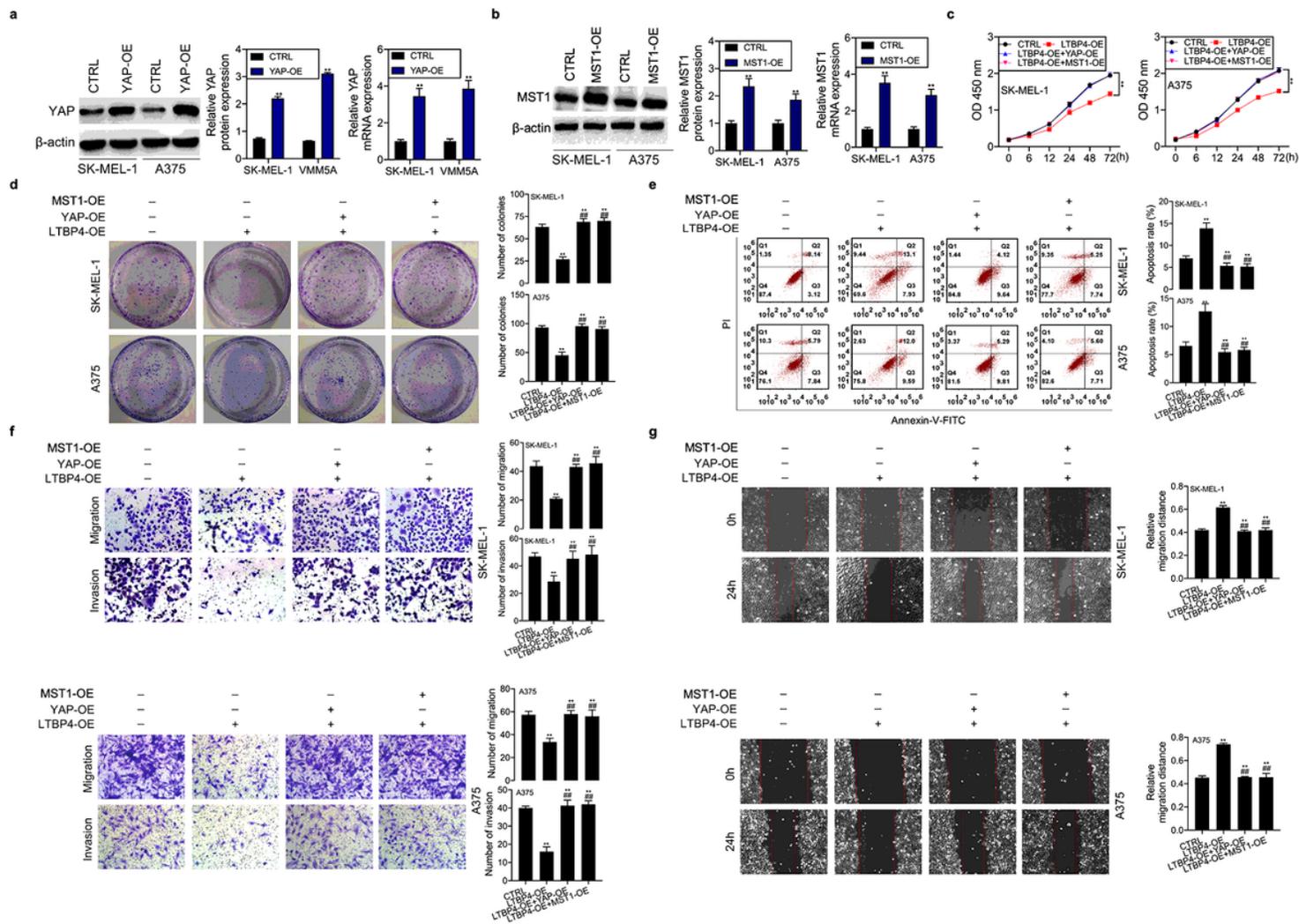


Figure 8

Overexpression of LTBP4 inhibited the proliferation, invasion and migration, and promoted the apoptosis of melanoma cells via Hippo-YAP signaling (a) The protein and mRNA levels of YAP in YAP expressing plasmids transfected SK-MEL-1 and A375 cells were detected by western blotting and RT-PCR assays. (b) The protein and mRNA levels of MST1 in MST1 expressing plasmids transfected SK-MEL-1 and A375 cells were detected by western blotting and RT-PCR assays. After LTBP4 expressing plasmids and YAP/or MST1 expressing plasmids were co-transfected into SK-MEL-1 and A375 cells, (c) the viability was detected by CCK-8 assay, (d) the proliferation was detected by colony formation assay, (e) the apoptosis level was detected by flow cytometry assay, (f) the migration and invasion were detected by transwell assay, and (g) the migration was detected by wound healing assay. β -actin was used as a load control. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. CTRL group and ## $P < 0.01$ vs. LTBP4-OE group.

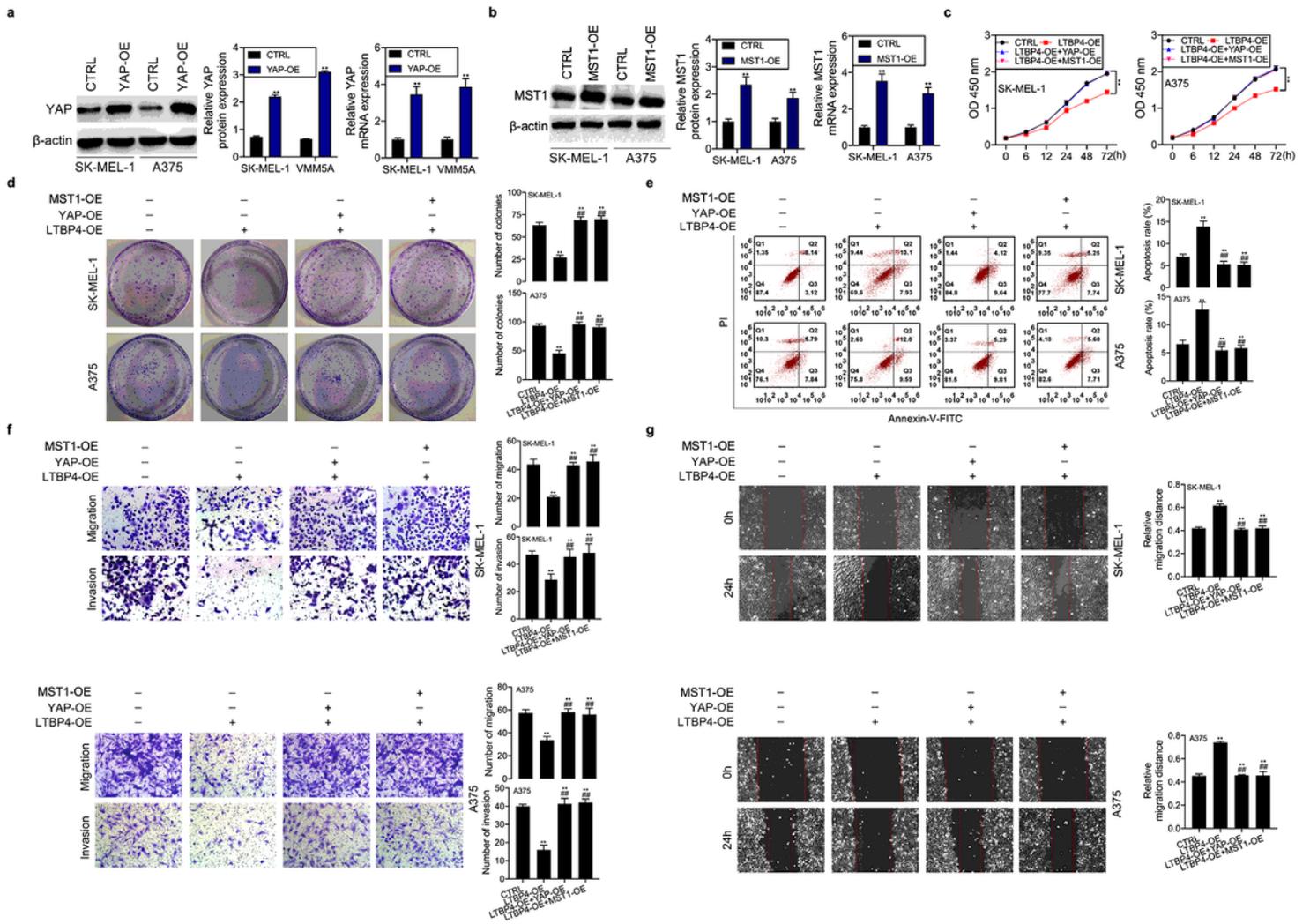


Figure 8

Overexpression of LTBP4 inhibited the proliferation, invasion and migration, and promoted the apoptosis of melanoma cells via Hippo-YAP signaling (a) The protein and mRNA levels of YAP in YAP expressing plasmids transfected SK-MEL-1 and A375 cells were detected by western blotting and RT-PCR assays. (b) The protein and mRNA levels of MST1 in MST1 expressing plasmids transfected SK-MEL-1 and A375 cells were detected by western blotting and RT-PCR assays. After LTBP4 expressing plasmids and YAP/or MST1 expressing plasmids were co-transfected into SK-MEL-1 and A375 cells, (c) the viability was detected by CCK-8 assay, (d) the proliferation was detected by colony formation assay, (e) the apoptosis level was detected by flow cytometry assay, (f) the migration and invasion were detected by transwell assay, and (g) the migration was detected by wound healing assay. β -actin was used as a load control. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. CTRL group and ## $P < 0.01$ vs. LTBP4-OE group.

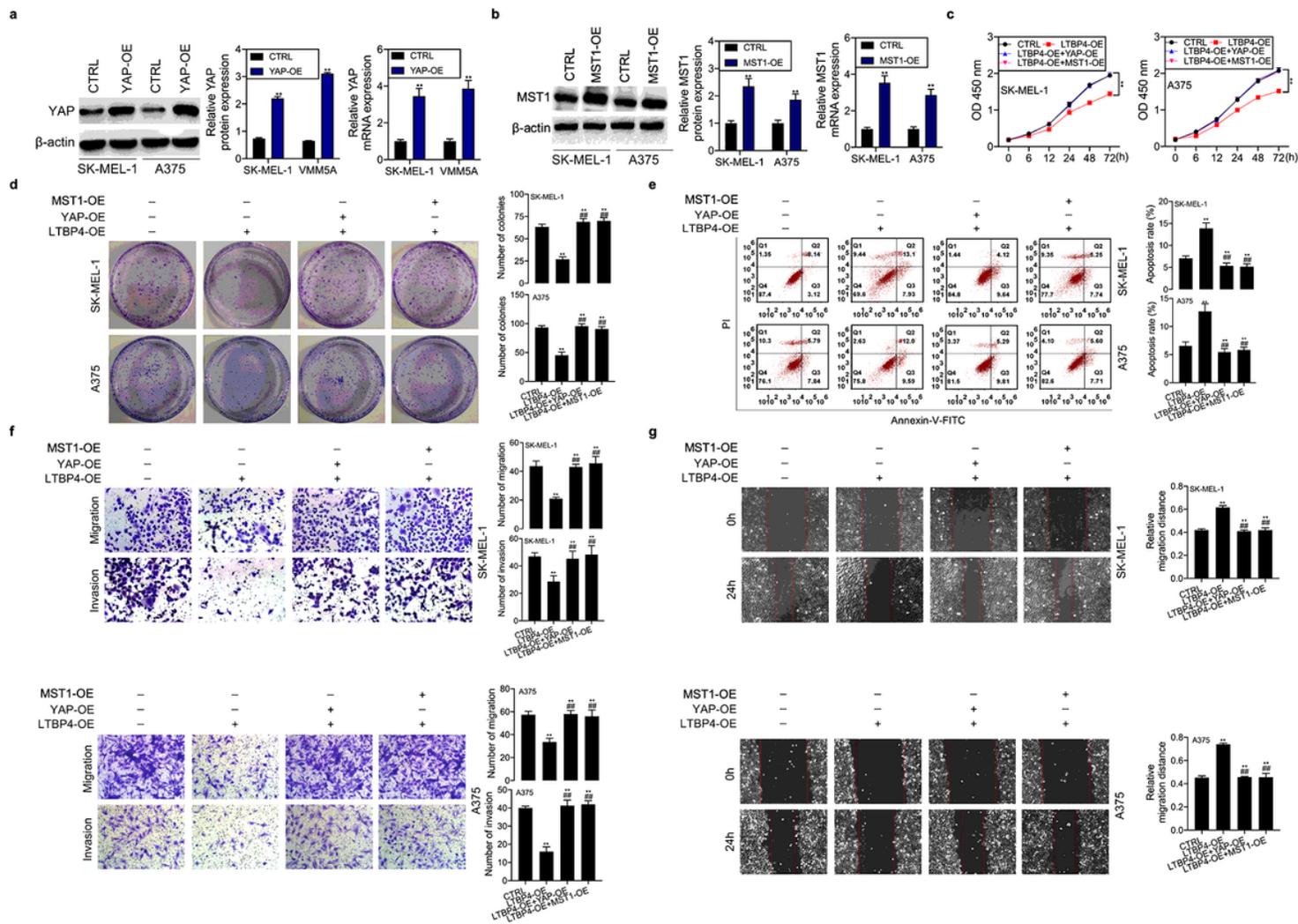


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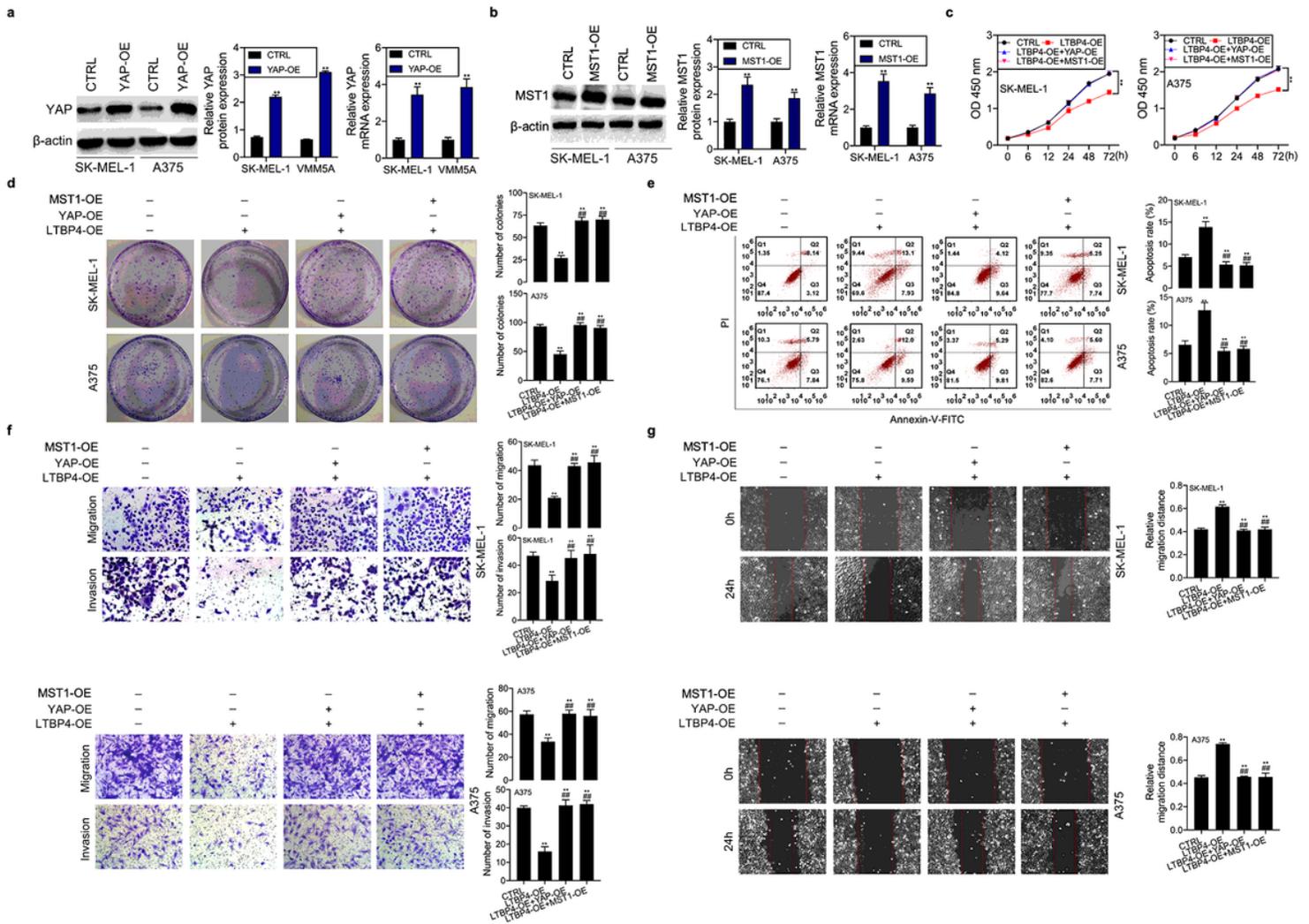


Figure 8

Overexpression of LTP4 inhibited the proliferation, invasion and migration, and promoted the apoptosis of melanoma cells via Hippo-YAP signaling (a) The protein and mRNA levels of YAP in YAP expressing plasmids transfected SK-MEL-1 and A375 cells were detected by western blotting and RT-PCR assays. (b) The protein and mRNA levels of MST1 in MST1 expressing plasmids transfected SK-MEL-1 and A375 cells were detected by western blotting and RT-PCR assays. After LTP4 expressing plasmids and YAP/or MST1 expressing plasmids were co-transfected into SK-MEL-1 and A375 cells, (c) the viability was detected by CCK-8 assay, (d) the proliferation was detected by colony formation assay, (e) the apoptosis level was detected by flow cytometry assay, (f) the migration and invasion were detected by transwell assay, and (g) the migration was detected by wound healing assay. β -actin was used as a load control. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. CTRL group and ## $P < 0.01$ vs. LTP4-OE group.

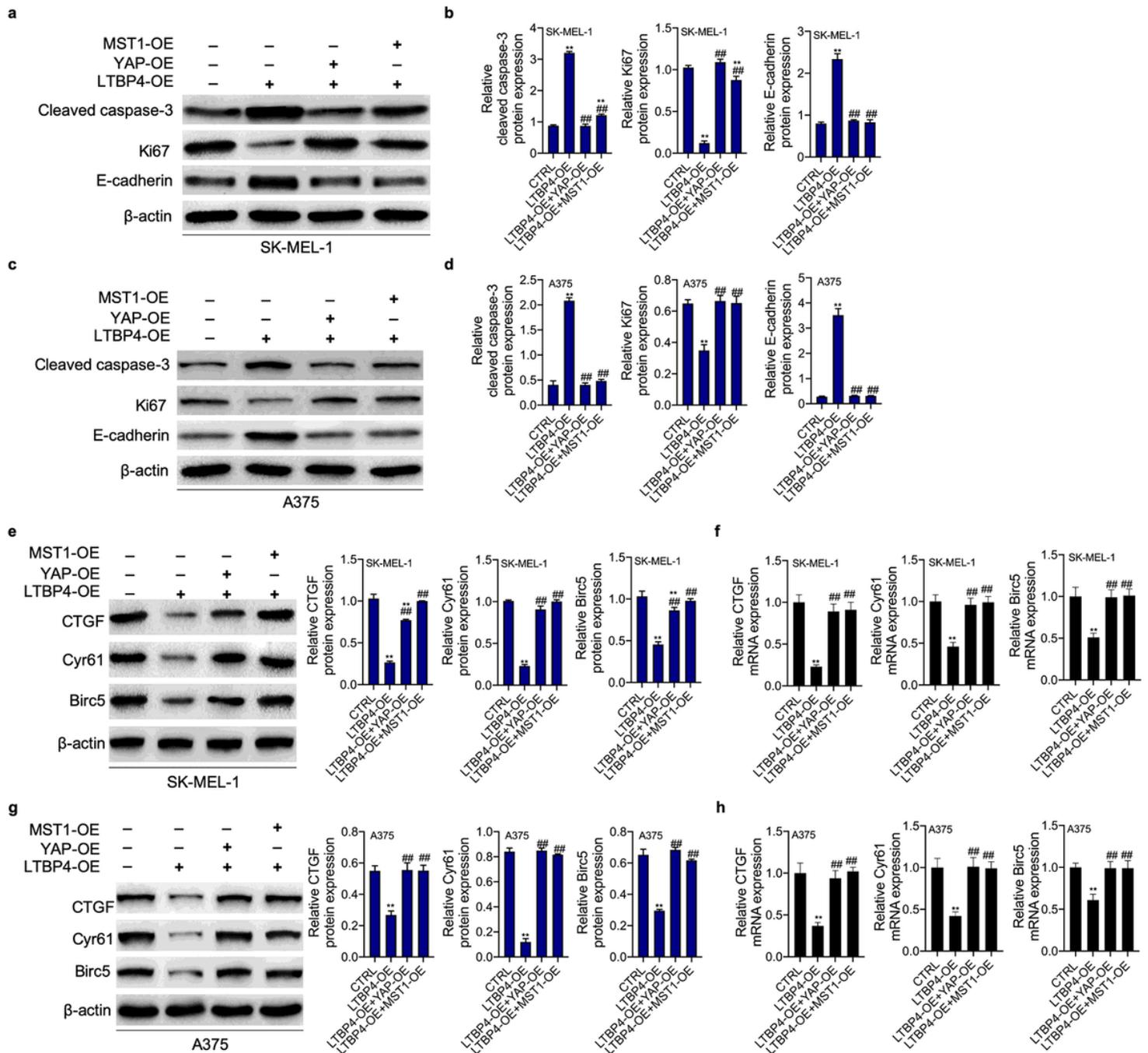


Figure 9

The functions of overexpression of LTBP4 regulating cleaved caspase-3, Ki67, E-cadherin, CTGF, Cyr61, and Birc5 were reversed by YAP OE or MST1 OE in melanoma cells. After LTBP4 expressing plasmids and YAP/or MST1 expressing plasmids were co-transfected into SK-MEL-1 and A375 cells, (a and b) the protein levels of cleaved caspase-3, Ki67, and E-cadherin in SK-MEL-1 cells were detected by western blotting assay, (c and d) the protein levels of cleaved caspase-3, Ki67, and E-cadherin in A375 cells were detected by western blotting assay, (e and f) the protein and mRNA levels of CTGF, Cyr61, and Birc5 in SK-MEL-1 cells were detected by western blotting and RT-PCR assays, and (g and h) the protein and mRNA levels of CTGF, Cyr61, and Birc5 in A375 cells were detected by western blotting and RT-PCR assays. β-

actin was used as a load control. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. CTRL group and ## $P < 0.01$ vs. LTBP4-OE group.

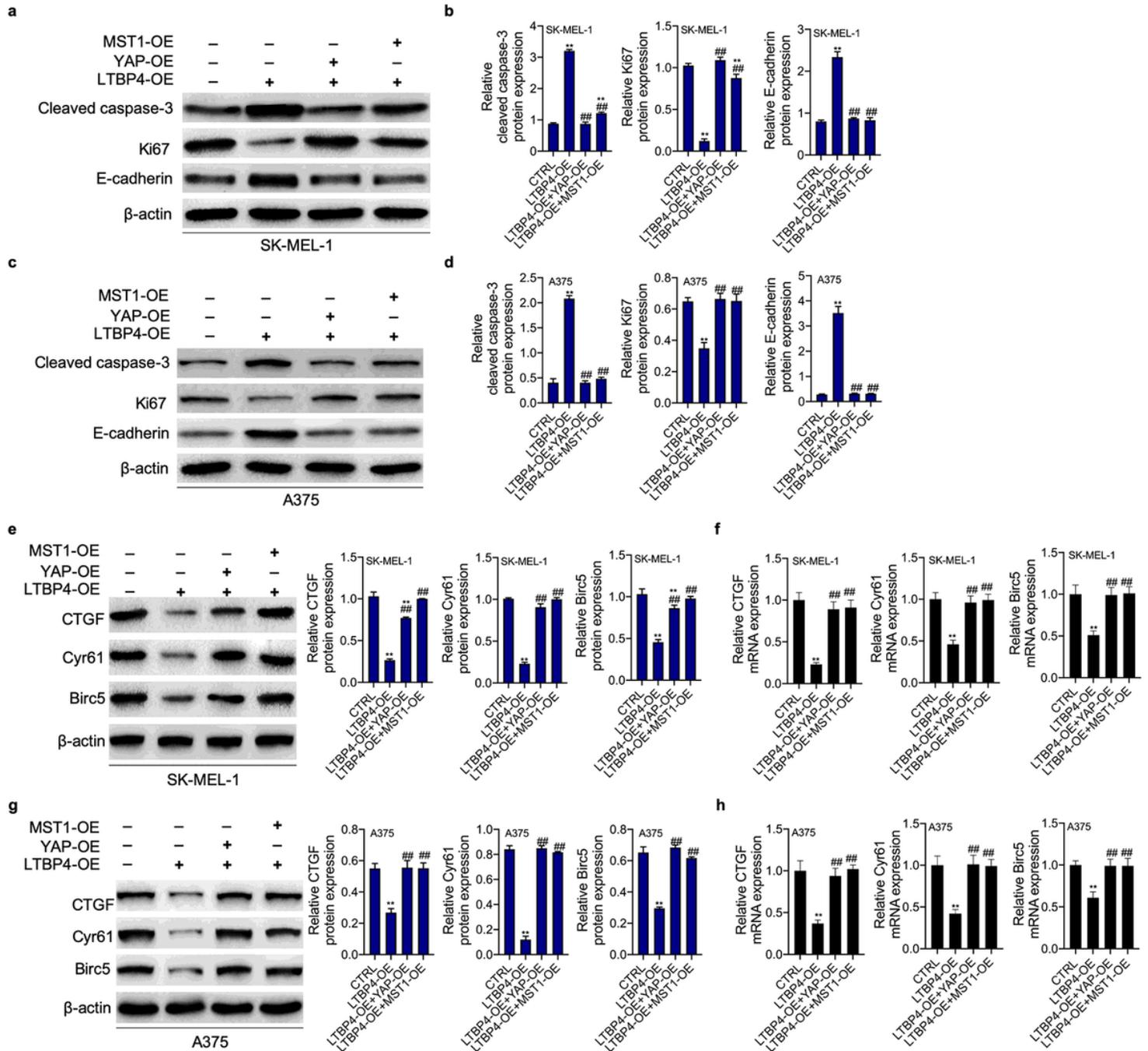


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MEL-1 cells were detected by western blotting and RT-PCR assays, and (g and h) the protein and mRNA levels of CTGF, Cyr61, and Birc5 in A375 cells were detected by western blotting and RT-PCR assays. β -actin was used as a load control. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. CTRL group and ## $P < 0.01$ vs. LTBP4-OE group.

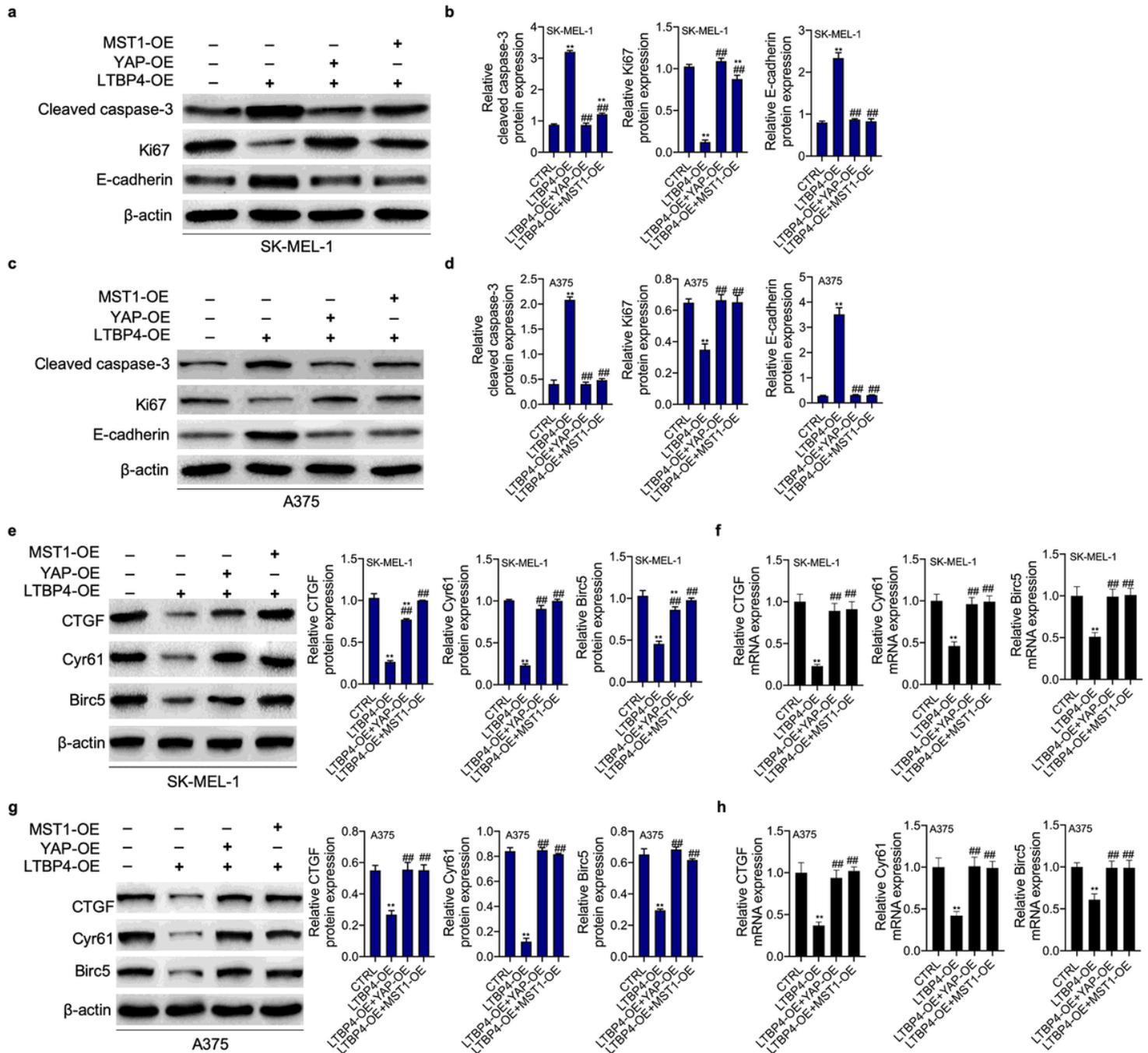


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blotting assay, (c and d) the protein levels of cleaved caspase-3, Ki67, and E-cadherin in A375 cells were detected by western blotting assay, (e and f) the protein and mRNA levels of CTGF, Cyr61, and Birc5 in SK-MEL-1 cells were detected by western blotting and RT-PCR assays, and (g and h) the protein and mRNA levels of CTGF, Cyr61, and Birc5 in A375 cells were detected by western blotting and RT-PCR assays. β -actin was used as a load control. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. CTRL group and ## $P < 0.01$ vs. LTBP4-OE group.

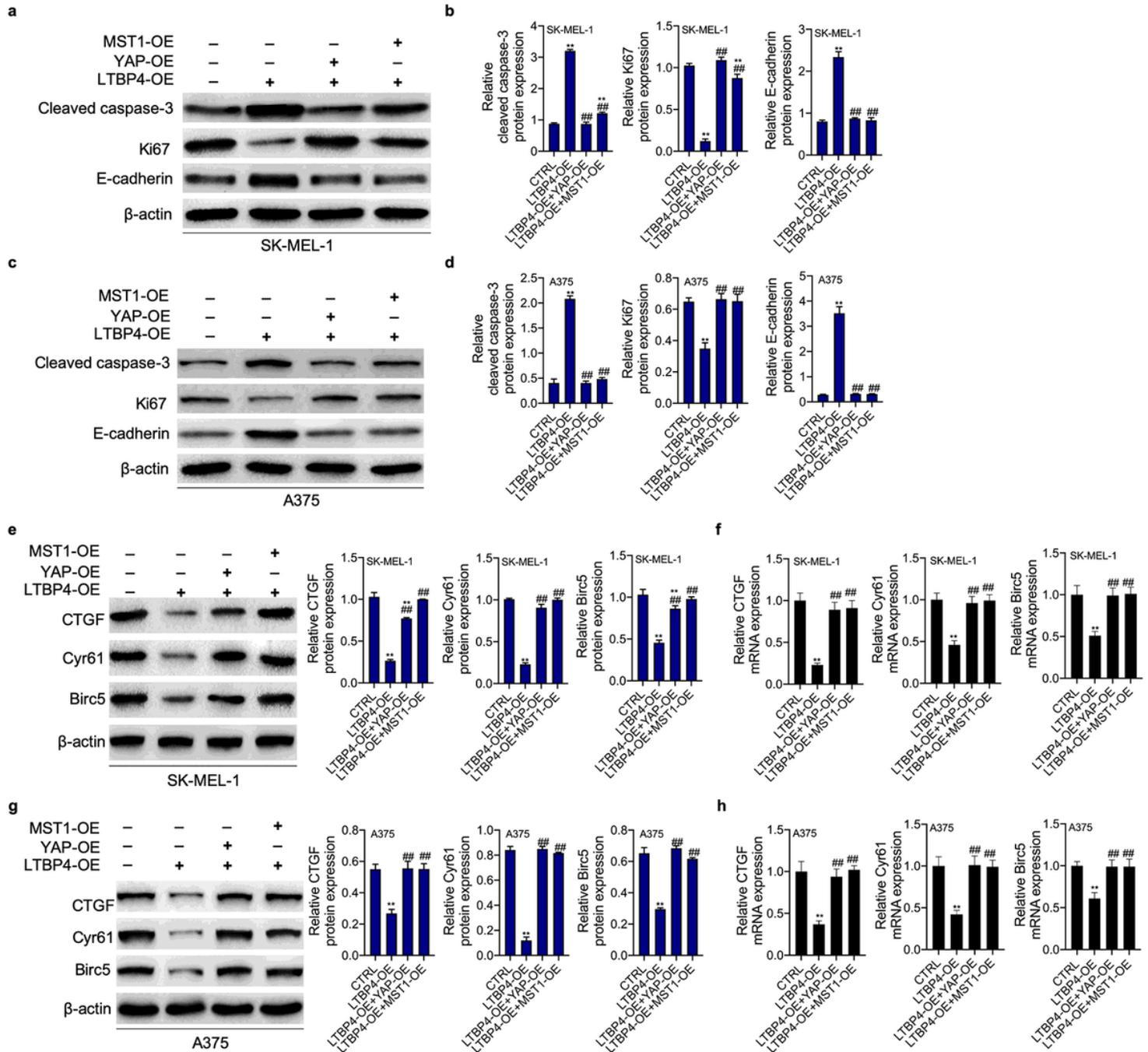


Figure 9

The functions of overexpression of LTBP4 regulating cleaved caspase-3, Ki67, E-cadherin, CTGF, Cyr61, and Birc5 were reversed by YAP OE or MST1 OE in melanoma cells After LTBP4 expressing plasmids and

YAP/or MST1 expressing plasmids were co-transfected into SK-MEL-1 and A375 cells, (a and b) the protein levels of cleaved caspase-3, Ki67, and E-cadherin in SK-MEL-1 cells were detected by western blotting assay, (c and d) the protein levels of cleaved caspase-3, Ki67, and E-cadherin in A375 cells were detected by western blotting assay, (e and f) the protein and mRNA levels of CTGF, Cyr61, and Birc5 in SK-MEL-1 cells were detected by western blotting and RT-PCR assays, and (g and h) the protein and mRNA levels of CTGF, Cyr61, and Birc5 in A375 cells were detected by western blotting and RT-PCR assays. β -actin was used as a load control. Data are presented as the mean \pm standard deviation. **P<0.01 vs. CTRL group and ##P<0.01 vs. LTBP4-OE group.

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