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Supporting Information

Title: Splice variant of growth hormone-releasing hormone receptor drives esophageal squamous cell carcinoma conferring a therapeutic target

Short title: GHRH-R splice variant as a therapeutic target

Author: Xiao Xiong*, Xiurong Ke*, Lu Wang*, Zhimeng Yao, Yi Guo, Xianyang Zhang, Yuping Chen, Chi Pui Pang, Andrew V. Schally, Hao Zhang

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2 [Contributed by Andrew V. Schally]

3 Please send only the initial correspondence to: [1]Dr Andrew V. Schally, Research
4 Service (151), Veterans Affairs Medical Center, 1201 NW 16th Street, Miami,
5 FL33125. E-mail: Andrew.Schally@va.gov

6

7 Subsequent correspondence and galley proof should be sent to:
8 Prof. Hao Zhang
Institute of Precision Cancer Medicine and Pathology, Department of Pathology, Jinan University Medical College, Guangzhou, Guangdong, China. Email: haozhang@jnu.edu.cn

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SI Materials and Methods

Cells and Cell Culture. The human ESCC cells HKESC-1, HKESC-2, HKESC-3, and 2 human immortalized esophageal epithelial cells (NE2 and NE083) were kindly provided by Dr. S.W. Tsao (University of Hong Kong, China). The human ESCC cells TE1, TE7 and TE12 cells were kindly provided by Dr. X.C. Xu (M.D. Anderson Cancer Center, Houston, TX, USA). The human ESCC cells KYSE140, KYSE150, KYSE510 were obtained from the tumor cell bank of the Chinese Academy of Medical Science. All cells were tested and authenticated in August 2013 using short tandem repeat validation analysis by the Cell Culture Service, Beijing Microread Genetics Co., Ltd. (Beijing, China). These ESCC cells were cultured in DMEM or RPMI 1640 medium supplemented with 10% FBS, 100 unit/mL of penicillin and 100 unit/mL of streptomycin at 37℃ in a humidified atmosphere with 5% CO_2. Immortalized NE2 and NE083 cells were cultured in Defined Keratinocyte-SFM (DK-SFM)/Epilife mixed medium (Life Technologies Gibco/BRL) (1). Hypoxic conditions in the indicated experiments were induced by putting the cells into a GasPak Pouch (BD Biosciences) for 24 h.

Generation of Stable Cell Lines. To generate stable cells overexpressing SV1, PFKM, p65, or silencing p65, cells were seeded onto 6-well dishes and grown to 80% confluence; the pcDNA3.1-SV1 vector, pcDNA3.1-PFKM vector, pReceiver-Lv201-p65, or psi-LVRU6GP-shp65, psi-LVRU6GP-shSV1 (sequences, shSV1#1, GGAGTTGTGGCTAGAGAGTCT; shSV1#2, GGGCCATAGCATCTCTATTGT) along with corresponding control vectors was transfected into TE1, KYSE140 or KYSE150 cells, respectively, using Lipofectamine 3000 (Life Technologies) according to the manufacturer's instructions. Stable transfectants were selected with puromycin (Sigma Aldrich, St Louis, MO) for 2 weeks. Resistant colonies were pooled and subcultured in the selection medium.

Animal Experiment. Once an individual tumor reached an average volume of 92 mm^3, the mice were randomized into five groups (n = 10). All groups were treated subcutaneously with 5 μg of the GHRH-R antagonist MIA-602 or with vehicle solution daily for 4 weeks. Tumor volumes were measured every week, and tumor mass was calculated by the following formula: volume = 0.5236 × length × $width^2$. Mice were killed after 4 weeks of treatment, and tumors were excised for paraffin block preservation.

Cell Viability Assays. Cell viability was analyzed by CCK-8 assay or monitored by the xCELLigence RTCA DP system (ACEA Biosciences). Cell Counting Kit-8 (MedChemExpress, New Jersey, USA) was used for CCK-8 assays. Cells were plated in 96-well plates at a density of 2000 cells in 100 μL medium per well, starved with medium without FBS for 24 h, followed by treatment with indicated concentrations of MIA-602 in medium containing 0.5% FBS for 48 h. Then 10 μL of the CCK-8 solution was added to each well of the plate and incubated at 37 °C for 2 h. The absorbance at 450 nm was measured to calculate the number of vital cells in each well. For Real-time cell analysis (RTCA), experiments were performed according to the supplier's instructions. Briefly, TE1 cells were seeded at a density of 3500 cells and KYSE140 cells at a density of 2000 cells in 100 μL medium in E-plates per well. The plates were locked into the device and incubated in at 37 ℃ in a humidified atmosphere with 5% CO_2. Cell viabilities were recorded every 15 min.

Colony-Formation Assays. Colony-formation assays were performed as described previously (5). Briefly, KYSE150 or KYSE140 cells were seeded into six-well plates in triplicate at a con- centration of 500 cells per well. After 24 h incubation, the cells were washed with PBS, and then RPMI 1640 (Gibco/Invitrogen) supplemented with either 2% FBS (Gibco/Invitrogen) with MIA-602 or vehicle solution was added. The medium was changed every 3 d. Cells were treated for a total of 9 d. The cell colonies then were fixed by methanol

and dyed with 0.1% crystal violet; the colonies that contained more than 50 cells were counted.

Cell Migration and Invasion Assay. Cell migration and invasion assays were conducted using the xCELLigence RTCA Analyzer (Roche Applied Science, Mannheim, Germany). Briefly, 150 μL of RPMI‑1640 supplemented with 10% FBS was added to the lower chamber in the CIM-16 plate (16-well, 8 μm pore filter) and 3×10^4 KYSE150 cells in 100 μL of serum-free RPMI-1640 were added to the upper chamber with or without Matrigel coating (BD Biosciences, Bedford, MA, USA). Cell index values that represented relative changes in electrical impedance on the underside of the 8 μm pore membrane were taken at a 15-minute interval.

Measurements of Glucose Uptake and Lactate Production.
Cells were seeded onto 12-well plates to allow attaching for 6-8 h and replaced with fresh complete medium or medium containing 5 μM MIA-602 or control vehicle. The medium was collected after 48 h incubation by centrifugation to remove the cells. Glucose uptake and lactate production were measured using the Glucose (GO) Assay Kit (Sigma Aldrich) and Lactate Assay Kit (Sigma Aldrich) according to the manufacturer's instruction, respectively.

Immunohistochemical Analysis. We performed immunohistochemistry (IHC) staining as previously described (2, 3). Briefly, 4 μm sections were cut from formalin-fixed, paraffin-embedded mice tumor tissues were underwent deparaffinization and rehydration, followed by endogenous peroxidase blocking, antigen retrieval. Sections of mice tissues incubated overnight at 4 ℃ with antibodies against Ki67 (Cat. ab16667; Abcam), Anti-PFKM (HPA002117; Sigma), Phospho-NF-κB p65 (#3033; Cell Signaling Technology), respectively. All sections were then incubated with the HRP-conjugated secondary antibodies at room temperature for 1.5 h, followed by incubation with 3, 3'-diaminobenzidine (DAB)

substrate to visualize positive staining. Nuclei were counterstained with hematoxylin. The percentage of positive staining cells was calculated by counting the number of positive staining cells based on hematoxylin-labeled nuclei, derived from ten random high-power fields (× 400). The mean of IOD was calculated by Image Pro Plus v.6.0 image analysis system. All sections were evaluated by two independent observers.

TNF-α Treatment and Immunofluorescence Assay. Cells were processed before being subjected to immunofluorescence analysis as described previously (4) and then were incubated with specific primary antibody against NF-κB p65 (#8242; Cell Signaling Technology) overnight at 4 °C, followed by Alexa Fluor 594 (red)–conjugated donkey anti-rabbit secondary antibody for 1 h in darkness. Nuclei were stained with DAPI. Images were captured using Zeiss Imager A2a fluorescence microscope.

Reverse Transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the cells or clinical specimens using TRIzol (Invitrogen). Four micrograms of RNA was reverse-transcribed using M-MLV Reverse Transcriptase (Invitrogen) with oligo-(dT) 20 primer (Invitrogen) according to the manufacturer's instructions. Then an equal amount of cDNA was amplified using an SYBR Green PCR amplification kit (Invitrogen) with the Applied Biosystems 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA) as described previously. The results were normalized to β-actin as an internal control. All reactions were run in triplicate. The cDNA was subjected to PCR with the following primers:

GHRH-R forward: 5'-ATGGGCTGCTGTGCTGGCCAAC-3'

GHRH-R reverse: 5'-TAAGGTGGAAAGGGCTCAGACC-3'

SV1 forward: 5'-CCACCCTCTCTGTTGCTCAG-3'

SV1 reverse: 5'-GTAAGATTCCTCCTCAGCCAGC-3'

PFKM forward: 5'-AATGGGCGGATCTTTGCCA-3'

PFKM reverse: 5'-TGGGGATTCGATGCTCAAAATCT-3'

PFKL forward: 5'-TCGACTGCAGGACCAATGTC-3'

PFKL reverse: 5'-CATGCGGTGCTCGAAATCAG-3'

PFKP forward: 5'-GACCTTCGTTCTGGAGGTGAT-3'

PFKP reverse: 5'-CACGGTTCTCCGAGAGTTTG-3'

HK2 forward: 5'-CGTCTACAAGAAACACCCCCATT-3'

HK2 reverse: 5'-ACCTCGCTCCATTTCTACCTTCA-3'

PKM forward: 5'-TCGCATGCAGCACCTGATT-3'

PKM reverse: 5'-CCTCGAATAGCTGCAAGTGGTA-3'

p65 forward: 5'-TGGCGAATGGCTCGTCTGTAGT-3'

p65 reverse: 5'-GGTCTTGGTGGTATCTGTGCTCCT-3'

β-actin forward: 5'-GAACCCCAAGGCCAACCGCGAGA-3'

β-actin reverse: 5'-TGACCCCGTCACCGGAGTCCATC-3'

Luciferase Assays. Luciferase assays were performed as described previously (1). In brief, cells were grown to 80% confluence in a 12-well plate and were then transiently transfected with 100 ng of luciferase reporter vector and 25 ng of pRLSV40 control vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h transfection, the cells were harvested and lysed in Luciferase Lysate Buffer (Promega, Madison, WI, USA); both firefly and Renilla luciferase activities were assayed using a dual-luciferase assay system (Promega). All experiments were performed in triplicate.

Immunoblotting. Immunoblotting was performed as described previously (5, 6). Briefly, the cells were lysed in RIPA buffer, and then equivalent amounts of the protein extracts were resolved using 10% SDS/PAGE and then transferred to a PVDF membrane. The membranes were blocked in 5% skim milk in TBS containing 0.1% Tween-20 (TBST) buffer and then incubated with the primary antibodies against anti-GHRH-R (ab28692; Abcam), Anti-PFKM (HPA002117; Sigma), NF-κB p65 (#8242; Cell Signaling Technology), Phospho-IKKα/β (#2694; Cell Signaling Technology), Phospho-NF-κB p65 (#3033; Cell Signaling

Technology), GAPDH (#5174; Cell Signaling Technology), IKKα/β (sc-7606; Santa Cruz Biotechnology), p-IκB-α (sc-101713; Santa Cruz Biotechnology), IκB-α (sc-371; Santa Cruz Biotechnology), at 4 °C overnight, respectively. After incubation with the primary antibodies, the secondary antibodies were added and incubated for 2 h at room temperature. The immunoreactive bands were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and were exposed to X-ray film (Eastman Kodak).

Gene Set Enrichment Analyses. Gene set enrichment analysis (GSEA) was performed to examine the association between gene sets and gene expression (7). Microarray data (accession No. GSE47404) were obtained from the NCBI's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and analyzed with GSEA software (version 2.0.13) (http://www.broadinstitute.org/gsea/index.jsp).

Statistical Analysis. All statistical analyses were performed using the SPSS 13.0 statistical software package (SPSS Inc.). The correlation between SV1 expression and clinicopathological features of ESCC patients was analyzed by the χ^2 test. Overall survival was estimated using the Kaplan–Meier method, and the difference in survival was evaluated using the log-rank test. Univariate and multivariate survival analysis was done with the Cox proportional hazards regression model. Comparisons between two groups were performed with a Student's *t* test, or paired *t* test, and comparisons among more than two groups were performed with one-way ANOVA with post hoc intergroup comparisons. All bar graphs show the mean ± SEM of at least three independent experiments. A *P* value of less than 0.05 was considered statistically significant.

SI Figures and Figure legends

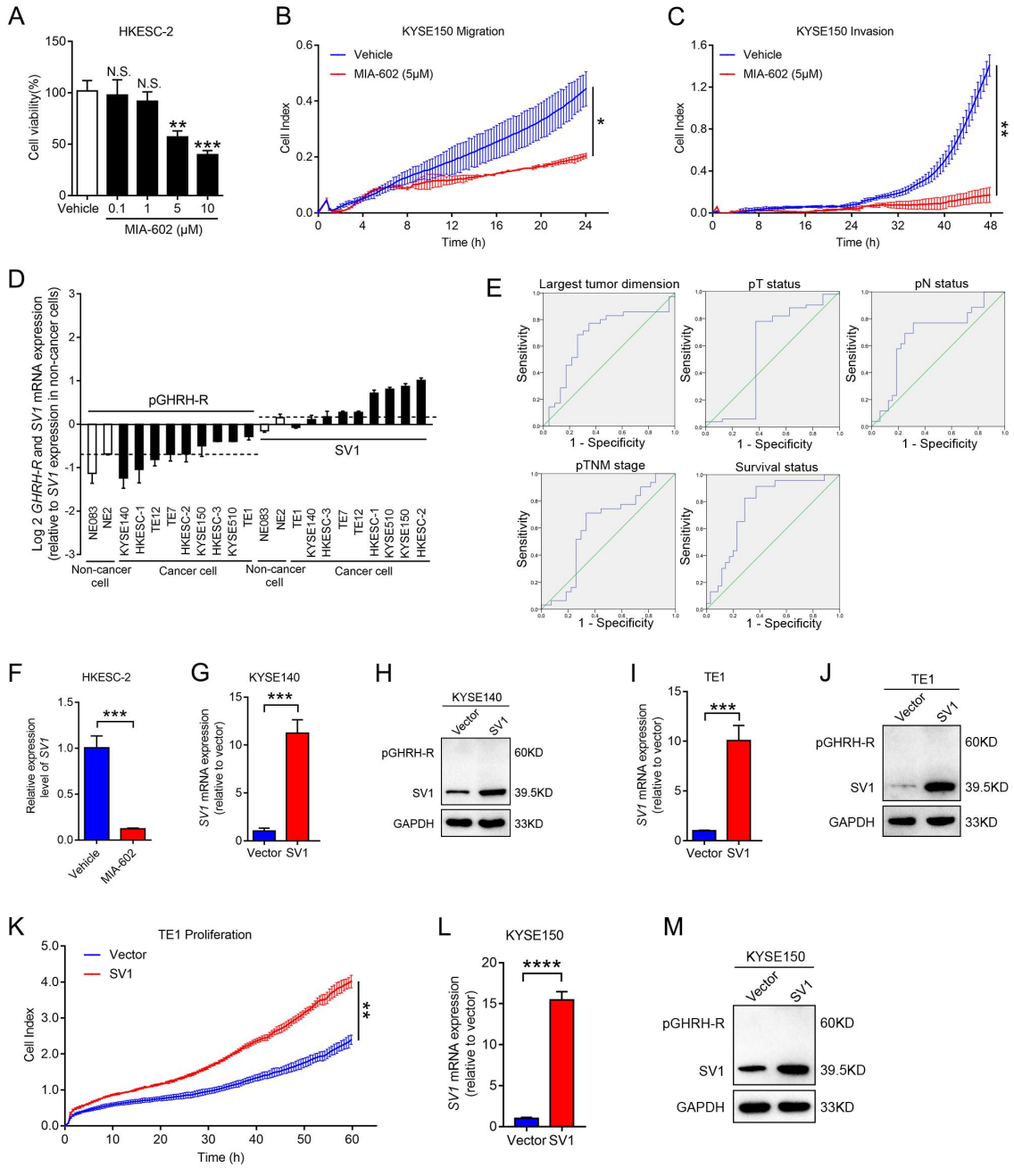

Fig. S1. MIA-602 inhibits ESCC cell progression through SV1. (A) The viability of HKESC-2 cells treated with MIA-602 (0.1, 1, 5, or 10 μM) or vehicle solution for 48 h was measured by CCK-8 assay. (B-C) Migration (B) and invasion (C) of KYSE150 cells treated with MIA-602 or vehicle were monitored by the xCELLigence RTCA DP system. Quantitative analysis of the cell index at 24 h (migration) and 48 h (invasion) are shown. (D) mRNA levels of *pGHRH-R* and *SV1* (relative to *SV1* expression in non-cancer cells) in 2 non-cancer cells (NE2, NE083)

(open bars) and 9 ESCC cells (filled bars) were analyzed by RT-qPCR. Dashed lines indicate the highest expression of *pGHRH-R* (-0.68) and *SV1* (0.15) in non-cancer cells. (E) The sensitivity and specificity for each clinical outcome were plotted. Clinical samples were split into groups with high (n = 28) and low (n = 30) *SV1* expression by ROC analysis. The blue trace represents ROC curve and the green trace represents diagonal reference line. (F) HKESC-2 cells were treated with MIA-602 or vehicle, levels of *SV1* were determined by RT-qPCR. (G-H) Expression of SV1 was measured by RT-qPCR (G) and immunoblotting (H) in KYSE140 cells transfected with SV1-overexpressing plasmid or control vector. (I-J) Expression of SV1 was measured by RT-qPCR (I) and immunoblotting (J) in TE1 cells transfected with SV1-overexpressing plasmid or control vector. GAPDH was used as an internal control. (K) Proliferation of SV1-overexpressing TE1 cells were monitored by the xCELLigence RTCA DP system. Quantitative analysis of the cell index at 60 h is shown. (L-M) Expression of SV1 was measured by RT-qPCR (L) and immunoblotting (M) in KYSE150 cells transfected with SV1-overexpressing plasmid or control vector. Error bars indicate SEM. N.S., not significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 by Student's *t* test (B-C, F-G, I, K-L) or one-way ANOVA with post hoc intergroup comparisons (A); n = 3 in each group.

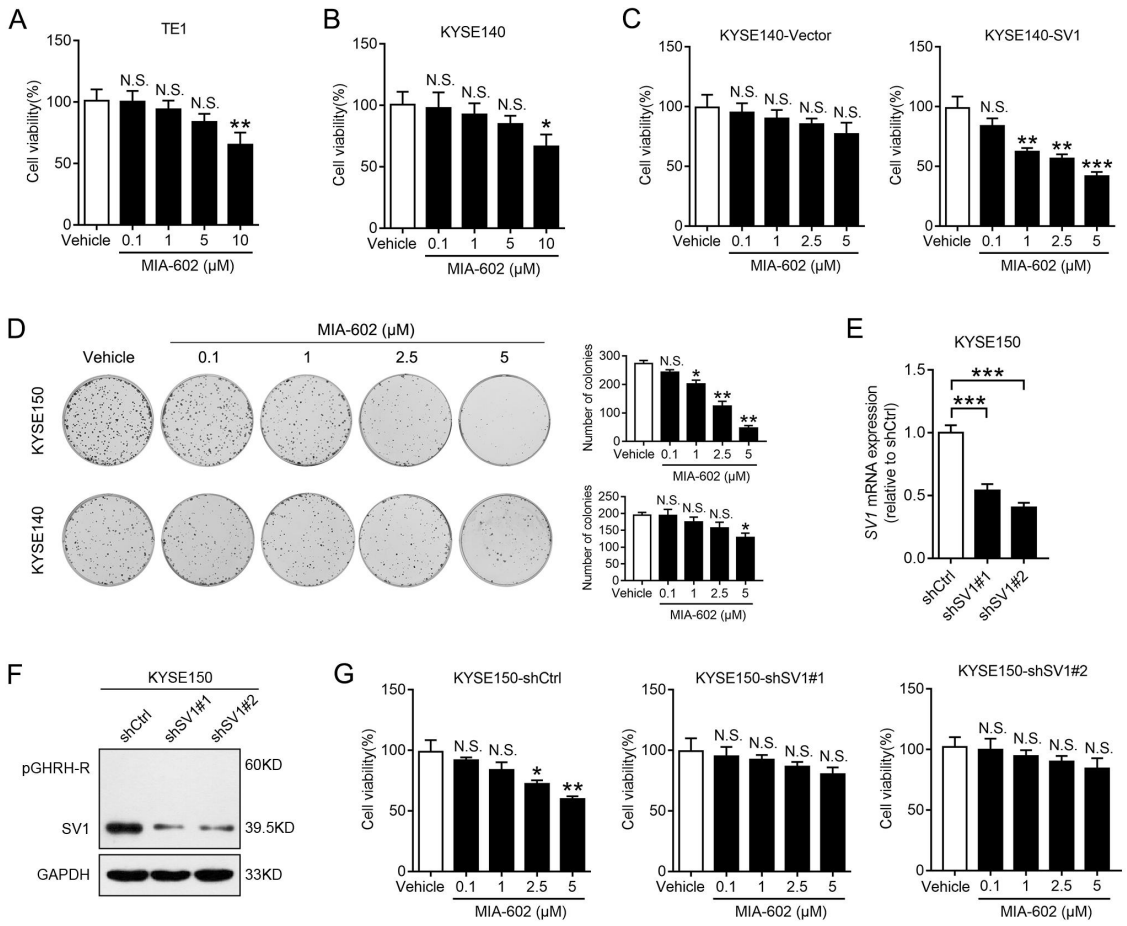

Fig. S2. SV1 mediates the inhibitory effects of MIA-602 in ESCC. (A-B) The viability of TE1 (A) and KYSE140 (B) cells treated with MIA-602 (0.1, 1, 5, or 10 μM) or vehicle solution for 48 h was measured by CCK-8 assay. (C) The viability of KYSE140 cells transfected with SV1-overexpressing plasmid or control vector treated with MIA-602 (0.1, 1, 2.5, or 5 μM) or vehicle solution for 48 h was measured by CCK-8 assay. (D) Clonogenicity of KYSE150 and KYSE140 cells treated with MIA-602 (0.1, 1, 2.5, or 5 μM) or vehicle solution was assessed by colony formation assay. Quantitative analysis of colony numbers is given on the right. (E-F) Expression of SV1 in SV1-knockdown KYSE150 cells were determined by RT-qPCR (E) and immunoblotting (F). (G) The viability of KYSE150 cells transfected with SV1-specific shRNA or control vector treated with MIA-602 (0.1, 1, 2.5, or 5 μM) or vehicle solution for 48 h was measured by CCK-8 assay. Error bars indicate SEM. N.S., not significant; *P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA with post hoc intergroup comparisons (A-E, G); n = 3 in each

group.

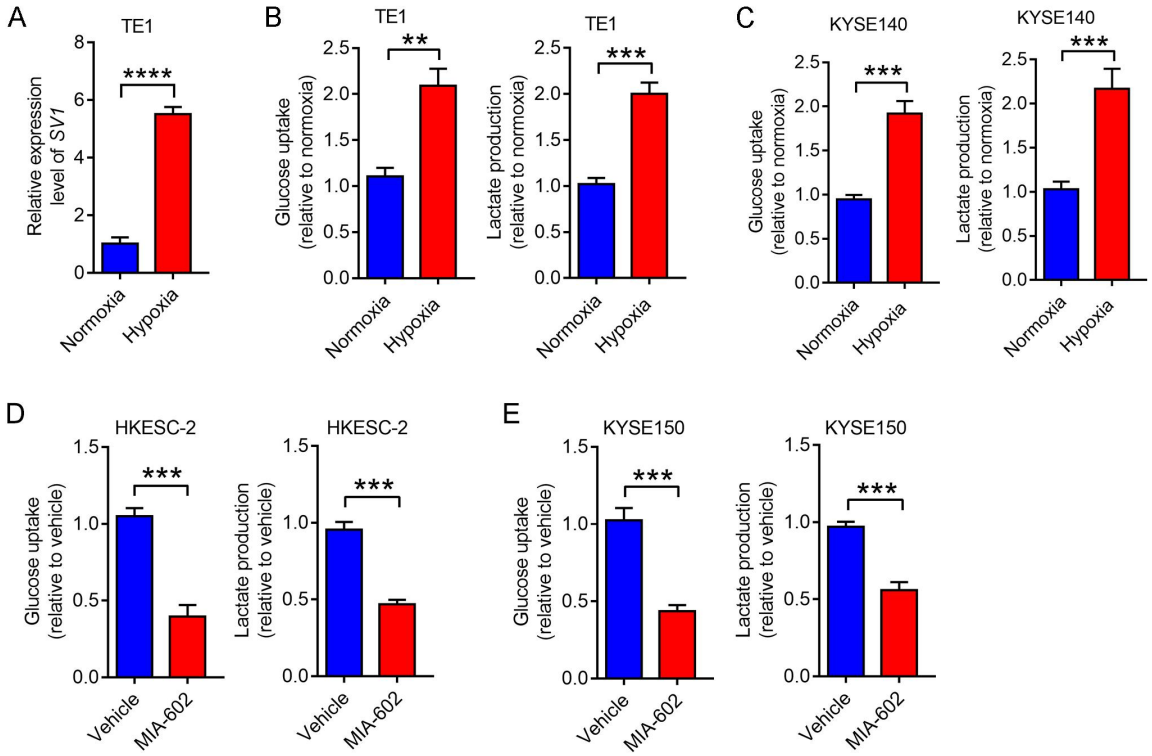

Fig. S3. SV1 is induced by hypoxia and enhances glycolysis. (A) Expression of *SV1* was measured by RT-qPCR in TE1 cells pre-treated at normoxia or hypoxia for 24 h. (B-C) Glucose uptake and lactate production were measured in TE1 (B) and KYSE140 (C) cells pre-treated at normoxia or hypoxia for 24 h. (D-E) Glucose uptake and lactate production were measured in HKESC-2 (D) and KYSE150 (E) cells treated with MIA-602 for 48 h. Error bars indicate SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by Student's t *t*est; n = 3 in each group.

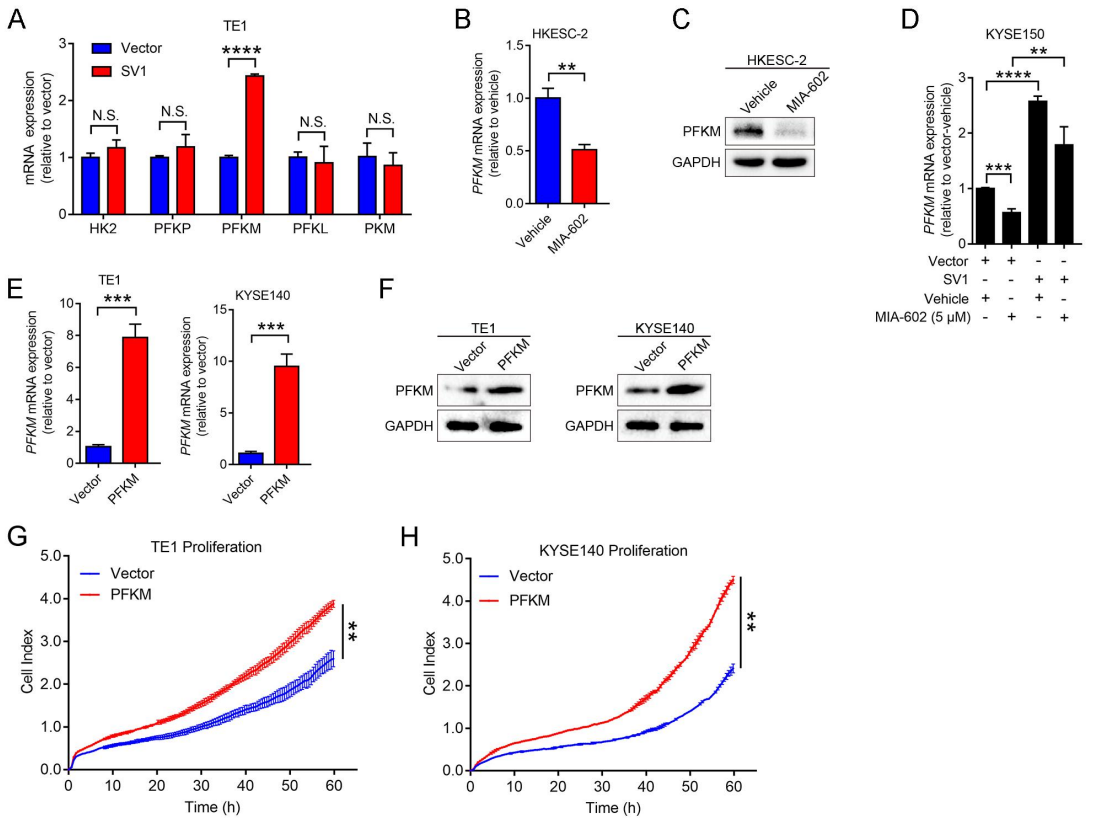

Fig. S4. SV1 targets PFKM to regulate glycolysis. (A) mRNA levels of 5 key glycolytic enzymes in SV1-overexpressing TE1 cells were determined by RT-qPCR. (B-C) Expression of PFKM in HKESC-2 cells treated with MIA-602 for 48 h was analyzed by RT-qPCR (B) and immunoblotting (C). GAPDH was used as an internal control. (D) Expression of PFKM in SV1-overexpressing cells treated with MIA-602 or vehicle were analyzed by RT-qPCR. (E-F) Expression of PFKM was measured by RT-qPCR (E) and immunoblotting (F) in TE1 and KYSE140 cells transfected with PFKM-overexpressing plasmid or control vector. GAPDH was used as an internal control. (G-H) Proliferation of PFKM-overexpressing TE1 (G) and KYSE140 (H) cells were monitored by the xCELLigence RTCA DP system. Quantitative analysis of the cell index at 60 h is shown. Error bars indicate SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by Student's *t* test (A-B, E, G-H) or one-way ANOVA with post hoc intergroup comparisons (D); n = 3 in each group.

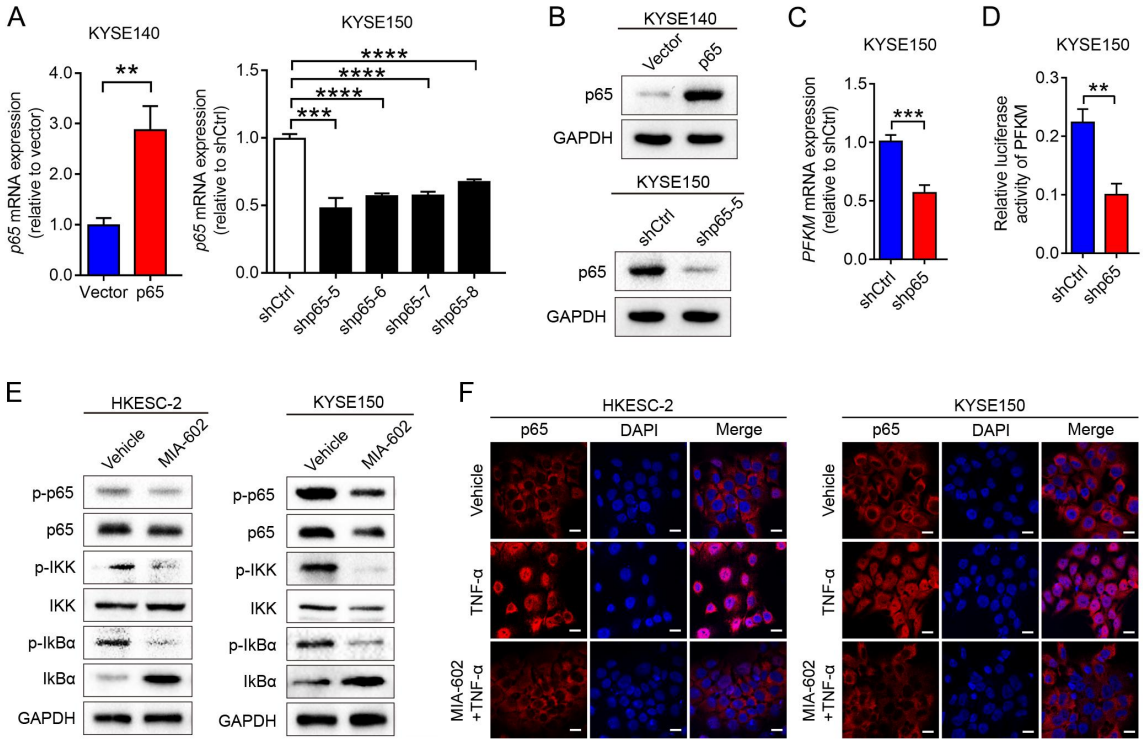

Fig. S5. MIA-602 suppresses NF-κB signaling. (A-B) Expression of p65 in p65-overexpressing KYSE140 cells and p65-knockdown KYSE150 cells were determined by RT-qPCR (A) and immunoblotting (B) GAPDH was used as an internal control. (C) mRNA level of *PFKM* in p65-knockdown cells were determined by RT-qPCR. (D) The PFKM luciferase reporter was transfected into KYSE150-shp65 cells and control shRNA cells, and the relative PFKM promoter activities were measured based on the luciferase activities. (E) HKESC-2 and KYSE150 cells were treated MIA-602 or vehicle before being harvested for immunoblot analyses of the labeled antigens. GAPDH was used as an internal control. (F) Subcellular localization of p65 (red) in HKESC-2 and KYSE150 cells, as analyzed by an immunofluorescence confocal assay. Nuclei were stained with DAPI (blue). Error bars indicate SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by Student's *t* test (A, Left, C-D) or one-way ANOVA with post hoc intergroup comparisons (A, Right); n = 3 in each group.

Table S1. Correlation between *SV1* expression and clinicopathological factors in ESCC patients

Clinicopathological factors	n	*SV1* expression		*P* value
		Low *SV1*, n (%)	High *SV1*, n (%)	
Patients	58	30 (51.7%)	28 (48.3%)	
Gender				
Male	37	19 (51.4%)	18 (48.6%)	0.940
Female	21	11 (52.4%)	10 (47.6%)	
Age (years)				
≤ 60	31	18 (58.1%)	13 (41.9%)	0.300
> 60	27	12 (44.4%)	15 (55.6%)	
Esophageal location				
Upper/Middle	49	25 (51.0%)	24 (49.0%)	0.802
Lower	9	5 (55.6%)	4 (44.4%)	
Histological differentiation				
Well	20	11 (55.0%)	9 (45.0%)	0.717
Moderately/Poorly	38	19 (50.0%)	19 (50.0%)	
Largest tumor dimension (cm)				
< 5	23	17 (73.9%)	6 (26.1%)	0.006
≥ 5	35	13 (37.1%)	22 (62.9%)	
pT status				
T1-T2	8	5 (62.5%)	3 (37.5%)	0.511
T3-T4	50	25 (50.0%)	25 (50.0%)	
pN status				
N0	32	22 (68.8%)	10 (31.3%)	0.004
N1-N3	26	8 (30.8%)	18 (69.2%)	
pTNM stage				
I-II	27	18 (66.7%)	9 (33.3%)	0.034
III	31	12 (38.7%)	19 (61.3%)	

pT, pathological tumor; pN, pathological nodal; pTNM, pathological tumor-node-metastasis.

Table S2. Univariate and multivariate Cox proportional hazards model predicting survival in ESCC patients

Clinicopathological factors	Univariate analysis		Multivariate analysis model 1		Multivariate analysis model 2	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Gender						
Male vs. Female	1.758 (0.693-4.461)	0.235	2.031 (0.759-5.436)	0.159	2.006 (0.759-5.303)	0.160
Age (years)						
≥ 60 *vs.* < 60	1.632 (0.711-3.744)	0.248	1.528 (0.633-3.689)	0.346	2.385 (0.994-5.723)	0.051
Esophageal location						
Lower *vs.* Upper/Middle	0.814 (0.242-2.746)	0.741				
Histological differentiation						
Moderately/Poorly *vs.* Well	1.088 (0.460-2.571)	0.847				
Largest tumor dimension (cm)						
≥ 5 *vs.* < 5	2.762 (1.024-7.447)	0.045			2.082 (0.754-5.750)	0.157
pT status						
T3-T4 *vs.* T1-T2	4.398 (0.586-32.997)	0.150	3.401 (0.444-26.052)	0.239		
pN status						
N1-N3 *vs.* N0	1.978 (0.865-4.522)	0.106				
pTNM stage						
III *vs.* I-II	3.526 (1.376-9.035)	0.009			3.621 (1.369-9.579)	0.010
SV1 expression						
High *vs.* Low	5.045 (1.866-13.640)	0.001	4.269 (1.547-11.775)	0.005		

HR, hazard ratio; CI, confidence interval

SI References

1. Dong H, *et al.* (2017) Reciprocal androgen receptor/interleukin-6 crosstalk drives oesophageal carcinoma progression and contributes to patient prognosis. *The Journal of pathology* 241(4):448-462.
2. Dong H, *et al.* (2017) PTPRO represses ERBB2-driven breast oncogenesis by dephosphorylation and endosomal internalization of ERBB2. *Oncogene* 36(3):410-422.
3. Gan J, *et al.* (2015) Dysregulation of PAK1 Is Associated with DNA Damage and Is of Prognostic Importance in Primary Esophageal Small Cell Carcinoma. *International journal of molecular sciences* 16(6):12035-12050.
4. Gan JF, *et al.* (2016) Growth hormone-releasing hormone receptor antagonists inhibit human gastric cancer through downregulation of PAK1-STAT3/NF-kappa B signaling. *Proceedings of the National Academy of Sciences of the United States of America* 113(51):14745-14750.
5. Tang Q, *et al.* (2013) Resveratrol-induced apoptosis is enhanced by inhibition of autophagy in esophageal squamous cell carcinoma. *Cancer letters* 336(2):325-337.
6. Wang L, *et al.* (2018) The oncogenic roles of nuclear receptor coactivator 1 in human esophageal carcinoma. *Cancer medicine* 7(10):5205-5216.
7. Yu J, *et al.* (2019) Detection of Exosomal PD-L1 RNA in Saliva of Patients With Periodontitis. *Frontiers in genetics* 10:202.