

E3 ubiquitin ligase MAGI3-mediated TAZ ubiquitination suppresses the non-small cell lung cancer occurrence and development by targeting the Wnt pathway

YE LIANG¹ and JIE ZHUANG²

¹Department of Allergy, Qingdao Integrated Chinese and Western Medicine Hospital, Qingdao, Shandong 266000, P.R. China;

²Department of Rehabilitation Medicine, The Third People's Hospital of Qingdao, Qingdao, Shandong 266041, P.R. China

Received April 20, 2026; Accepted August 31, 2026

DOI: 10.3892/ijo.2026.5939

Abstract. Membrane associated guanylate kinase WW and PDZ domain containing 3 (MAGI3) functions as a tumor suppressor in lung cancer, but its role and mechanism in non-small cell lung cancer (NSCLC) progression remain unclear. MAGI3 expression was detected in NSCLC tissues (immunohistochemistry) and cell lines (western blotting). Tet-on inducible MAGI3 overexpression was used to assess effects on NSCLC cells via Cell Counting Kit-8, Transwell, colony/sphere formation, β -Galactosidase staining and flow cytometry. Ubiquitination/half-life assays analyzed MAGI3-TAZ (transcriptional co-activator with PDZ-binding motif) interaction. *In situ* and xenograft tumor models evaluated tumor growth and metastasis. The Cancer Genome Atlas data, clinical samples, and cell lines showed downregulated MAGI3 in NSCLC, correlating with poor prognosis. MAGI3 overexpression reduced cell viability, clonogenicity, sphere formation, migration and invasion; induced G1 phase arrest (reduced CyclinD1) and cellular senescence. MAGI3 specifically promoted TAZ ubiquitination via PDZ domain-mediated interaction, which subsequently blocked β -catenin nuclear translocation, dampened Wnt signaling components (DVL2/phosphorylated GSK3 β), and reduced TCF/LEF transcriptional output. Reconstitution of TAZ reversed MAGI3-mediated inhibition of Wnt pathway markers (cMYC, c-JUN, WISP1 and PPAR δ). *In vivo*, MAGI3 overexpression suppressed tumor growth and lung metastasis, while MAGI3 silencing increased metastatic nodules. The present study identified that MAGI3, as a suppressor in NSCLC progression, repressed the Wnt pathway-including

TAZ degradation, impaired β -catenin nuclear translocation, and diminished TCF/LEF transcription-thereby restraining tumorigenesis and metastasis of NSCLC.

Introduction

Lung cancer is the main cause of death worldwide, with an estimated nearly 236,740 new cases and 127,750 deaths based on 2022 statistics (1). Taking into account of histological features, lung cancer consists of small cell lung cancer and non-small cell lung cancer (NSCLC), among which NSCLC subtype accounts for 85% (2). Despite advances in NSCLC diagnosis and treatment, five-year survival remains below 20% and more than 90% of patients succumb to metastasis (3). Thus, illuminating the mechanism of NSCLC occurrence and development is key to identify the potential drug targets that interfere with NSCLC.

The transcriptional co-regulators YAP/TAZ is considered to be considered as pro-oncogenic factors that actuate a variety of oncogenic traits (4). Additionally, prior studies have stated that the regulating the TAZ stability is crucial for tumorigenesis and resistance including lung cancer (5,6), indicating that TAZ may be a druggable target for NSCLC treatment. Wnt signaling pathway is a conserved signaling cascade that modulates embryonic development and cancer progression (7). For NSCLC, aberrant activation of Wnt pathway largely functions as the tumorigenesis, resistance and prognosis (8). Furthermore, a previous study has also illustrated the interplay between TAZ and Wnt pathway, showing that TAZ is required for Wnt-induced tumorigenesis (9). Altogether, it raised a possibility that TAZ/Wnt feedback loop may exhibit causative impacts in NSCLC tumorigenesis, and thus understanding the mechanisms by which regulates TAZ/Wnt feedback loop becomes critical.

To seek the additional associated-genes, membrane associated guanylate kinase WW and PDZ domain containing 3 (MAGI3), located at the 1p13.2, is an E3 ubiquitin ligase that catalyzes the polyubiquitination of substrates (10). A previous study has stated that MAGI3 tends to restrain the malignant tumors via negatively modulating the Wnt pathway (11). On the other hand, Wang *et al* (12) have supplied evidence that MAGI3 boosts the cMYC ubiquitination to degradation. However, the

Correspondence to: Dr Jie Zhuang, Department of Rehabilitation Medicine, The Third People's Hospital of Qingdao, 29 Yongping Road, Licang, Qingdao, Shandong 266041, P.R. China
E-mail: zhuangjie@qdu.edu.cn

Key words: membrane associated guanylate kinase WW and PDZ domain containing 3, TAZ, ubiquitination and degradation, Wnt pathway, metastasis, non-small cell lung cancer

functions of MAGI3 in NSCLC cells and whether MAGI3 targets other substrates to involve in the NSCLC progression are entirely unknown.

In the current study, the evidence that MAGI3, as a novel upstream modulator, promotes TAZ ubiquitination and degradation, was disclosed. Additionally, the mechanism that MAGI3 suppresses the Wnt signaling by reducing the stability of TAZ, which in turn leads to the NSCLC occurrence and development, was identified.

Materials and methods

Clinical samples. Tumor specimens and corresponding adjacent non-tumor lung tissues were acquired from patients with NSCLC who underwent cancer resection. Samples were diagnosed by pathologists and stored at the liquid nitrogen. Moreover, formalin-fixed, paraffin-embedded (FFPE) NSCLC tissues, and paired adjacent non-tumor lung tissues were collected to investigate the clinicopathological exploration. The present study was approved (approval no. 202528) by the Ethics Committee of Qingdao Integrated Chinese and Western Medicine Hospital (Qingdao, China) and conducted in consistent with the declaration of Helsinki. Informed consent was obtained from all participants. Among the 30 patients with NSCLC included, there were 15 male and 15 female patients, with a median age of 44 years (range, 27-61 years), as detailed in Table I.

Cell culture. Human NSCLC cell lines (A549, PC9, H1155, SW900, H460, H1975, H1650, H23 and H1299) and human normal lung cell line (Beas-2b) were used in the present study. A549 (RRID: CVCL_0023; male, lung, derived from adenocarcinoma) and PC9 (RRID: CVCL_B260; female, lung, derived from adenocarcinoma) were purchased from Procell Life Science & Technology Co., Ltd. in March 2023. H460 (RRID: CVCL_0459; male, lung, derived from large cell carcinoma), H1975 (RRID: CVCL_1511; female, lung, derived from adenocarcinoma), H1650 (RRID: CVCL_1483; male, lung, derived from adenocarcinoma), H23 (RRID: CVCL_1547; male, lung, derived from adenocarcinoma), H1299 (RRID: CVCL_0060; male, lung, derived from non-small cell lung carcinoma), H1155 (RRID: CVCL_1456; male, lung, derived from small cell lung cancer) and SW900 (RRID: CVCL_1973; male, lung, derived from squamous cell carcinoma) were obtained from the American Type Culture Collection (ATCC) in January 2023. The normal lung cell line Beas-2b (RRID: CVCL_0168; male, lung, derived from normal bronchial epithelium) was purchased from Procell Life Science & Technology Co., Ltd. in February 2023. All cell lines were authenticated by the suppliers using short tandem repeat (STR) profiling, and STR profiles were confirmed to match the ATCC or DSMZ databases with >90% identity. The STR authentication reports for A549 (male) and PC9 (female) are provided as supplementary information. No cell line used in the present study is listed in the International Cell Line Authentication Committee database of misidentified or contaminated cell lines. All cells were cultivated in RPMI-1640 medium (Procell Life Science & Technology Co., Ltd.) containing 2.05 mM L-glutamine, 10 mM HEPES and 1 mM sodium pyruvate, and supplemented with 10% (v/v) heat-inactivated fetal bovine

serum (FBS; Gibco; Thermo Fisher Scientific, Inc.; Australian origin) and 1% penicillin-streptomycin (100 U/ml penicillin, 100 µg/ml streptomycin, Gibco; Thermo Fisher Scientific, Inc.). Cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂. All experiments were performed within 15 passages after thawing. All lines were screened for mycoplasma contamination immediately after the second post-thaw passage and before each functional experiment using the GMyc-PCR Mycoplasma Detection Kit (cat. no. 40601; Shanghai Yeasen Biotechnology Co., Ltd.). Only mycoplasma-negative cultures were expanded for experimental use. Wnt agonist HY-114321 (MedChemExpress) was dissolved in DMSO and used at a final concentration of 0.5 µM for 48 h, based on preliminary dose-response assays. Control cells received an equal volume of DMSO (final concentration ≤0.1%).

Plasmid constructs and generation of the Tet-On-inducible stable cell lines. Lenti-Tet-on overexpression MAGI3 plasmid (Tet-MAGI3) and its corresponding control plasmid (tet-NC) were constructed by Genomeditech Co., Ltd. For MAGI3 gene overexpression, A549 and PC9 cells with doxycycline (DOX) inducible expression of MAGI3 were proceeded by lentivirus transfection as previously described (13). The A549 and PC9 cells with stable overexpression of MAGI3 were detected by western blotting and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The PDZ-binding motif mutant of TAZ (TAZ-ΔPDZBM) was generated by site-directed mutagenesis using the wild-type TAZ plasmid as template. Specifically, the conserved C-terminal SSWV motif was mutated to S S A A (tryptophan W and valine V were substituted with alanine A) to abolish the PDZ domain-mediated interaction. Additionally, the PDZ domain-deleted MAGI3 mutant (MAGI3-ΔPDZ) was constructed by PCR-based deletion of the PDZ domain-encoding region. All constructs were verified by DNA sequencing. The sequences for shRNA (shMAGI3 and shNC) and small interfering RNA (siRNA) (siTAZ and siNC) used for knockdown/rescue experiments are listed in Table SI.

Immunohistochemistry (IHC) assay. After FFPE tissues incised into 4-µm sections, the slides were placed in the oven and baked for 20 min, dewaxed with xylene and hydrated. Subsequently, the slides were immersed in protease K to antigen repair. After washing, the slides were exposed to 3% hydrogen peroxide, followed by incubating with 10% goat serum (cat. no. 16210064; Gibco; Thermo Fisher Scientific, Inc.) to block at room temperature for 10 min. Primary antibodies including MAGI3 (1:50; cat. no. NBP1-81266), TAZ (1:50; cat. no. NBP1-88511; both from Novus Biologicals, LLC), anti-cMYC (1:200; cat. no. ARG40636), anti-c-JUN (1:300; cat. no. ARG55192) anti-WISP1 (1:200; cat. no. ARG42929) and anti-PPARδ (1:200; cat. no. ARG65761; Arigo Biolaboratories) were added for incubation, and then HRP-labelled IgG (1:800; cat. no. MF756-01; Mei5 Biotechnology Co., Ltd.) was added. The sections were dyed with 3, 3'-diaminobenzidine (DAB) and visualized by a light microscopy (BX43; Olympus Corporation). Immunostaining was quantified using the H-score system. For each section, five random fields (magnification, x400) within tumor areas (avoiding necrosis and mucus) were evaluated independently by two pathologists

Table I. Association between clinical parameters of patients with non-small cell lung cancer and MAGI3 expression.

Variable	Total	Expression level of MAGI3 (n=30)		P-value
		Low	High	
Age, years				>0.9999
<44	16	8	8	
≥44	14	7	7	
Sex				0.7150
Female	15	7	8	
Male	15	8	7	
Tumor size, cm				0.1213
<2	10	3	7	
≥2	20	12	8	
Tumor node metastasis stage				0.0502
I/II	10	2	8	
III/IV	20	13	7	
Lymph node metastasis				0.0502
Negative	10	8	2	
Positive	20	7	13	

MAGI3, membrane associated guanylate kinase WW and PDZ domain containing 3.

blinded to clinical data. Intensity was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong), and the percentage of positive cells (0-100%) was recorded. H-score was computed as Σ (intensity x percentage) and averaged between the two readers, giving a range of 0-300. Background staining was managed by using a negative control (PBS instead of primary antibody) and standardized acquisition settings; only specific brown precipitates within tumor cells were scored, while diffuse nonspecific signals were excluded. Discrepancies >10% between scorers were resolved by discussion, with a third pathologist as arbiter if needed.

Cell Counting Kit-8 (CCK-8) assay. Tet-On-inducible MAGI3-expressing A549 and PC9 cells (Tet-MAGI3) and their control cells (Tet-NC) (1×10^6 cells) were cultured in RPMI-1640 medium with or without 0.5 $\mu\text{g/ml}$ of DOX. Then, CCK-8 reagent (10 $\mu\text{l/well}$; MedChemExpress) was added and incubated at 37°C for 2 h, the absorbance was detected at 450 nm by microplate reader (Infinite EPlux; Tecan Group, Ltd.).

Transwell assay. Tet-On-inducible MAGI3-expressing A549 and PC9 cells (1×10^6 cells) were seeded and treated with or without 0.5 $\mu\text{g/ml}$ of DOX. After induction for 48 h, cells were re-suspended in serum-free RPMI-1640 medium and transferred (1×10^5 cells) to the upper chamber of invasion or migration Transwell plate (8- μm pore size; BD Biosciences). After incubation at 37°C for 24 h (migration) or 48 h (invasion), A549 and PC9 cells in the lower chambers (containing RPMI-1640 medium with 10% FBS as chemoattractant) were stained with 0.1% crystal violet at room temperature for 15 min, and the numbers of cells was counted under a light microscope (BX43; Olympus Corporation).

Colony formation assay. Tet-On-inducible MAGI3-expressing A549 and PC9 cells (1×10^3 cells) were cultivated into medium with or without 0.5 $\mu\text{g/ml}$ of DOX treatment. Following incubation, cells were then cultured in fresh medium for 2 weeks. Colonies of A549 and PC9 cells were fixed in 4% paraformaldehyde at room temperature for 15 min and then dyed with 0.1% crystal violet at room temperature for 15 min for visualization. Colonies were defined as clusters of at least 50 cells and were counted manually by two independent observers under a light microscope (BX43; Olympus Corporation); the mean of the three counts was used for analysis.

Sphere formation assay. As previously described (14), Tet-On-inducible MAGI3-expressing A549 and PC9 cells (2×10^3 cells) in different groups were cultivated in 6-well plates with ultralow attachment. Until the culture of 10 days, the forming spheres were collected, digested with pancreatin, and resuspended in serum-free RPMI-1640 medium. Subsequently, the dissociated single cells were seeded into 6-well plates as above mentioned. The number of spheres was captured by microscope (BX43; Olympus Corporation).

Cell cycle analysis. Tet-On-inducible MAGI3-expressing A549 and PC9 cells (1×10^6 cells) were collected and fixed with 75% precooled ethanol at 4°C overnight. Following centrifugation (1,000 x g at 4°C for 5 min), PI staining solution (0.5 ml) and RNase A (100 μl) was added and incubated against light following the manufacturer's instructions (Shanghai YaJi Biological Technology Co., Ltd.) at 37°C for 30 min. Next, flow cytometry was employed to determine the cell cycle in each group (BD Biosciences) using a BD FACSCanto II

flow cytometer, and the data were analyzed using ModFit LT software (version 5.0; Verity Software House).

Senescence associated β -Galactosidase staining analysis. Tet-On-inducible MAGI3-expressing A549 and PC9 cells (1×10^6 cells) were cultured in 6-well-plate overnight. After washing, senescence of cells was detected using with β -Galactosidase staining kit (Beyotime Institute of Biotechnology). The staining-positive cells were captured under microscope (BX43; Olympus Corporation).

Co-Immunoprecipitation (Co-IP) assay. 293T and A549 cells were lysed in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS; Beyotime Institute of Biotechnology) supplemented with 1 mM phenyl-methyl-sulfonyl fluoride and a protease inhibitor cocktail, and then cell lysates were incubated with anti-MAGI3, anti-IgG and Input antibodies (anti-MAGI3; 1:100; cat. no. NBP2-17210; Novus Biologicals, LLC; normal rabbit IgG isotype control, 1:100, cat. no. 2729, Cell Signaling Technology, Inc.; and Input, corresponding to 10% of the total cell lysate processed in parallel without immunoprecipitation) at 4°C overnight. Afterwards, protein A agarose beads (20 μ l of a 50% slurry per 500 μ l of cell lysate) were added to cell lysate incubated with antibodies. Following incubation, the beads were washed and boiled in SDS loading buffer. The immunoprecipitated proteins were determined by western blotting. To validate the specificity of the MAGI3-TAZ interaction, 293T cells were co-transfected with Flag-MAGI3 together with HA-tagged wild-type TAZ (HA-TAZ-WT) or HA-tagged PDZ-binding motif mutant TAZ (HA-TAZ- Δ PDZBM) using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) with 2 μ g of each plasmid per well of a 6-well plate. Transfection was performed at 37°C for 6 h, after which the transfection medium was replaced with complete medium, and the subsequent experiments were carried out 48 h later. Cell lysates were immunoprecipitated with anti-Flag (MAGI3) antibody, and the precipitated TAZ was detected by western blotting using anti-HA antibody. For the reciprocal validation, HA-TAZ was co-transfected with Flag-tagged wild-type MAGI3 or PDZ-deleted MAGI3 mutant (MAGI3- Δ PDZ), followed by IP with anti-HA and WB with anti-Flag.

Protein half-life detection. Tet-On-inducible MAGI3-expressing A549 and PC9 cells were treated with cycloheximide (CHX, 100 μ g/ml). After culturing for 0, 2, 4 and 6 h, cells were collected for protein extraction and western blotting. The relative expression of TAZ was adopted to evaluate the half-life of TAZ.

Ubiquitination assay. Tet-On-inducible MAGI3-expressing A549 cells were transfected with plasmid constructs including HA-tagged ubiquitin and then were administrated with 10 μ M MG-132 for 6 h. Whole cell lysates were utilized for IP with anti-TAZ overnight. The TAZ ubiquitination was evaluated by immunoblotting with anti-ubiquitin (1:1,000; cat. no. ARG54630; Arigo Biolaboratories) antibody. To confirm the specificity of MAGI3-induced TAZ ubiquitination, A549 cells were co-transfected with HA-Ubiquitin, Flag-MAGI3, and either Flag-tagged TAZ-WT or Flag-tagged

TAZ- Δ PDZBM. After MG132 treatment for 6 h, cell lysates were immunoprecipitated with anti-Flag antibody, and ubiquitination levels were detected by immunoblotting with anti-HA antibody.

RT-qPCR assay. Total RNA was extracted with TRIzol Reagent (Tiangen Biotech Co., Ltd.). Following, cDNA was synthesized and real-time PCR was conducted by One-step reverse transcription PCR kit (Tiangen Biotech Co., Ltd.). All procedures were performed according to the manufacturer's instructions. Reverse transcription was carried out at 50°C for 30 min, followed by enzyme inactivation at 95°C for 3 min. β -actin was termed as internal control for quantification of MAGI3. Thermocycling conditions were as follows: 95°C for 3 min; 95°C for 10 sec $\rightarrow$ 60°C for 30 sec $\rightarrow$ 72°C for 30 sec, 40 cycles; 72°C for 5 min. β -actin (ACTB; cat. no. NM_001101.5; NCBI) served as the internal reference. Primer sequences, expected amplicon sizes, annealing temperatures and GenBank accession numbers are provided in Table SII. Relative gene expression was calculated using the $2^{-\Delta\Delta C_q}$ method (15).

Western blotting. Protein was separated and electrically transferred to PVDF membrane. Following blocking with 5% skimmed milk at room temperature for 2 h, the membranes were incubated overnight at 4°C with anti-cyclinE1 (1:1,000; cat. no. ab33911), anti-p16 (1:1,000; cat. no. ab270058; both from Abcam), anti-cyclinB1 (1:3,000), anti-cyclinD1 (1:1,000; cat. no. ARG52923), anti-cMYC (1:1,000; cat. no. ARG40636), anti-c-JUN (1:2,000; cat. no. ARG55192), anti-WISP1 (1:2,000; cat. no. ARG42929), anti-PPAR δ (1:2,000; cat. no. ARG65761), anti-p21 (1:1,500; cat. no. ARG57928; all from Arigo Biolaboratories), anti-p53 (1:1,000; cat. no. 9282S; Cell Signaling Technology, Inc.), phosphorylated (p)-p53 (p53 phosphorylated at Ser15; 1:1,000; cat. no. ARG52352; Arigo Biolaboratories), MAGI3 (1:2,000; cat. no. NBP2-17210), anti-TAZ (1:1,000; cat. no. H00006901-M12; both from Novus Biologicals, LLC), anti-DVL2 (1:1,000; cat. no. ab129181; Abcam), anti-phospho-GSK3 β (Ser9) (1:1,000; cat. no. ab131097; Abcam) and anti-GSK3 β (1:2,000; cat. no. ab93926; Abcam). After washing, anti-Rabbit IgG-HRP (1:8,000; cat. no. MF756-01; Mei5 Biotechnology Co., Ltd.) were incubated for 2 h. Immunoblots were visualized by the enhanced chemiluminescence (ECL) kit (Tanon Science and Technology Co., Ltd.), and images were captured by Tanon 5200 (Tanon Science and Technology Co., Ltd.). Total protein was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with 1 mM phenyl-methyl-sulfonyl fluoride and a protease inhibitor cocktail, and the protein concentration was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). A total of 30 μ g of protein was loaded per lane and separated on 10% SDS-PAGE gels. Densitometric analysis was performed using ImageJ software (version 1.53a; National Institutes of Health).

Nuclear and cytoplasmic fractionation. Subcellular fractionation was performed to isolate nuclear and cytoplasmic protein fractions from A549 cells harboring a Tet-On-inducible MAGI3 overexpression system. The separation was carried out using a commercial nuclear/cytosol extraction kit

(Beyotime Institute of Biotechnology) following the supplier's recommended protocol. The resulting protein extracts from both compartments were subsequently analyzed by immunoblotting using specific primary antibodies: anti- β -catenin (1:1,000; cat. no. ab32572; Abcam), anti-Lamin B1 (1:2,000; cat. no. ab16048; Abcam; employed as a nuclear marker), and anti- β -actin (1:5,000; cat. no. ab8226; Abcam; used as a cytoplasmic marker).

Luciferase reporter assay. A549 cells with stable Tet-On-inducible MAGI3 expression were seeded into 24-well plates and transiently co-transfected with either TOP-Flash or FOP-Flash luciferase reporter constructs (MilliporeSigma), along with the pRL-TK *Renilla* luciferase internal control plasmid (Promega Corporation), using Lipofectamine 3000 (Invitrogen; Thermo Fisher scientific, Inc.). At 24 h post-transfection, the cells were exposed to 0.5 μ g/ml DOX or left untreated for an additional 4 h. Firefly and *Renilla* luciferase activities were then quantified with the Dual-Luciferase Reporter Assay System (Promega Corporation).

H&E staining. Tumor and lung tissues were fixed with 4% paraformaldehyde at 4°C for 24 h, embedded in paraffin, and cut into 4- μ m slides. Afterwards, the slices were dewaxed, rehydrated, and stained with H&E at room temperature, with hematoxylin applied for 5 min followed by eosin for 2 min. Images were captured by microscope (BX43, Olympus Corporation).

Lung cancer models. For tumor model *in situ*, the U6 promoter-sgRNA-EFS-Cas9-2A-Cre (pSECC) lentiviral vector was constructed by LanDM Biotech. The sgRNA sequences (sgMAGI3 and sgGFP) are provided in Table SII. Afterwards, lentiviral vectors were transfected with 293T cells to obtain the lentiviruses. Transfection was carried out at 37°C for 48 h using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Lentiviral particles were harvested from the culture supernatant at 48 and 72 h post-transfection, clarified by centrifugation (1,000 $\times$ g at 4°C for 5 min) and filtered through a 0.45- μ m membrane. For *in vitro* transduction, the cells were infected at a multiplicity of infection (MOI) of 20. Transduction was performed for 24 h, and the subsequent experiments were carried out 48 h later. Transduced cells were selected with 2 μ g/ml puromycin for 7 days. Kras-LSL-G12D mice were purchased by Shanghai Model Organisms Co., Ltd. Mice were housed under specific pathogen-free conditions at 22 $\pm$ 2°C and 50 $\pm$ 10% relative humidity, under a 12-h light/dark cycle, with free access to standard laboratory chow and sterile water. Mice were anesthetized with isoflurane (3%) before intratracheal infection. According to previous study, mice were infected intratracheally with 25,000 TU of lentiviruses (16). In brief, 5 mice were infected with sg-GFP-pSECC, as control; and 5 mice with sg MAGI3-pSECC to evaluate the influences of MAGI3 on lung after 8 weeks of infection.

For xenograft tumor model *in vivo*, Tet-On-inducible MAGI3-expressing A549 and PC9 cells (3 $\times$ 10⁶ cells) resuspended in 100 μ l of serum-free RPMI-1640 medium were subcutaneously implanted into the flank of female BALB/c-nu/nu mice (6-8 weeks, median age 7 weeks). A total of 7 days later, mice were divided into 2 group based on the

diet either or with 625 mg/kg DOX or lacking DOX (Harlan Laboratories). Tumor size and weights were monitored weekly for 4 weeks. *In vivo* metastasis assays, mice injection with Tet-On-inducible MAGI3-expressing A549 and PC9 cells were continue fed with DOX food or lacking DOX food for 8 weeks. Following deep anesthesia, mice were euthanized by cervical dislocation, and then tumor and lung tissues were collected for subsequent experiments. All animal experiments were conformed to the animal care and use guidelines and approved by the Institutional Animal Care and Use Committee of Ethics Committee of Beijing Viewsolid Biotechnology Co., Ltd. (approval no. VS2124A0023). All the operations followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and ARRIVE Guidelines pertaining to animal experimentation.

Bioinformatic analysis. To understand the MAGI3 expression in NSCLC, The Cancer Genome Atlas (TCGA) database (<https://portal.gdc.cancer.gov/>) from NSCLC was adopted. Boxplot was adopted to visualize the MAGI3 expression using the R package ggplot2. The differential gene was analyzed by R package Deseq2 (Love MI, Huber W and Anders S: Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15: 550, 2014; <https://bioconductor.org/packages/DESeq2/>) with a strand of 'fold change >2 and Padj <0.05'. Additionally, the relationship between MAGI3 expression and survival was analyzed using R packages 'survival' and R package 'survminer' based on TCGA database. The prognosis value of MAGI3 was detected by Kaplan-Meier plotter.

Statistical analysis. Data represent the mean $\pm$ standard deviation (SD) and were analyzed using SPSS 23.0 (IBM Corp.). Normality was examined with the Shapiro-Wilk test ($n < 50$) or Kolmogorov-Smirnov test ($n \geq 50$); homogeneity of variances was checked with Levene's test. Data from two groups were compared using the two-tailed unpaired Student's test. Multiple group comparisons were performed using a one-way ANOVA followed by Tukey's post-hoc test (equal variances) or Games-Howell test (unequal variances). $P < 0.05$ was considered to indicate a statistically significant difference. For survival analyses, the proportional hazards assumption was evaluated using cox.zph (Therneau TM and Grambsch PM: Modeling Survival Data: Extending the Cox Model. New York: Springer, 2000; <https://cran.r-project.org/package=survival>). When violated, we applied the Renyi-type supremum test and weighted log-rank tests as primary inferential tools, as detailed in the Bioinformatic analysis section. The association between MAGI3 expression (dichotomized as high vs. low) and clinicopathological variables was evaluated using Fisher's exact test, as the expected cell counts in >20% of cells were ≤ 5 for several variables (tumor size, TNM stage, and lymph node metastasis), violating the assumptions of the Chi-square test.

Results

MAGI3 is downregulated in NSCLC and relevant to prognosis. To determine the roles of MAGI3 on NSCLC progression, its expression was firstly predicted based on TCGA database. As presented in Fig. 1A, MAGI3 expression in the Tumor group

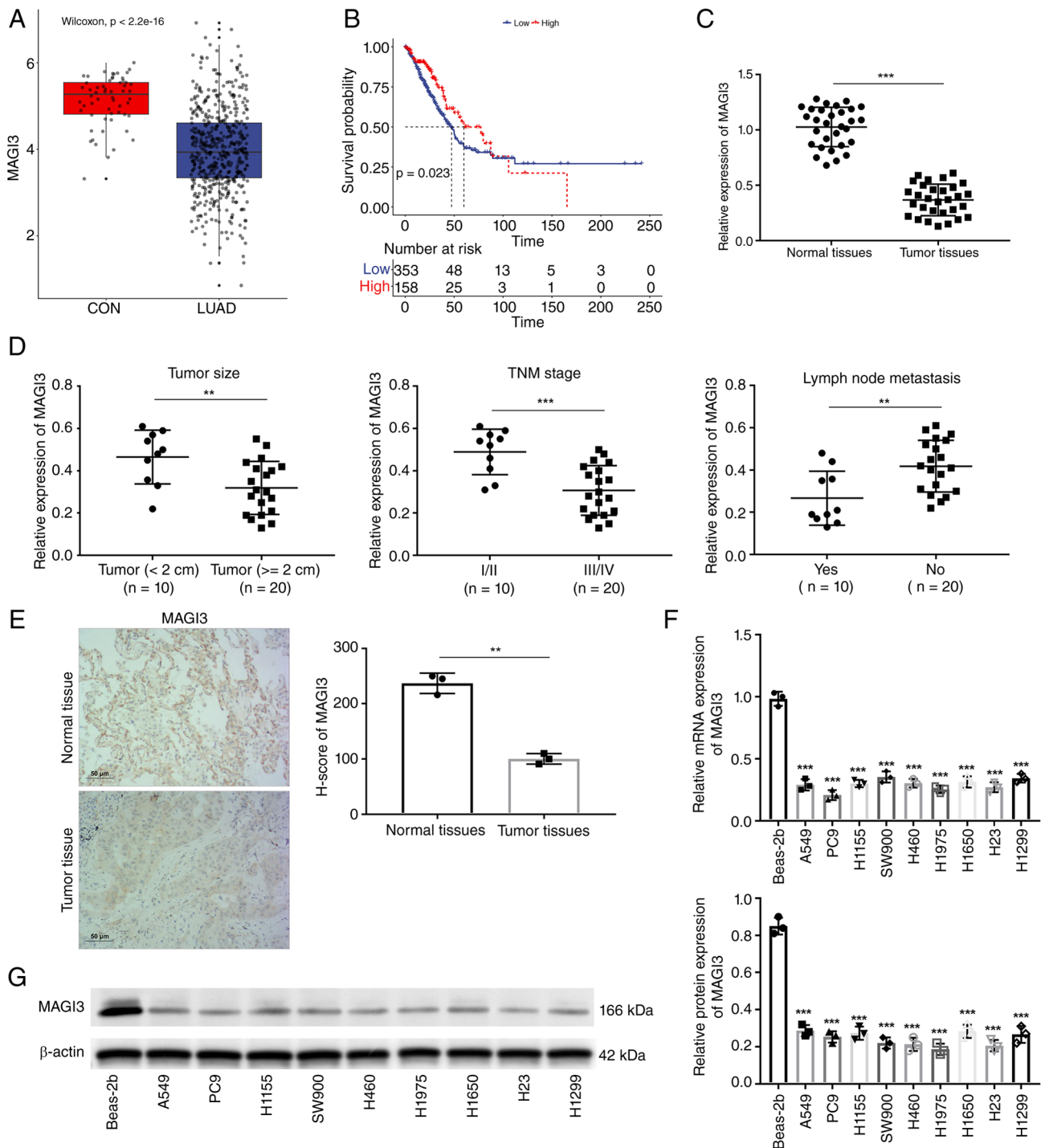


Figure 1. MAGI3 is downregulated in NSCLC and relevant to prognosis. (A) TCGA database presented that MAGI3 expression in the Tumor group was lower than of Control group. (B) Kaplan-Meier survival curves for patients with NSCLC stratified by MAGI3 expression level (high vs. low) based on TCGA data. The curves were compared using the Renyi-type supremum test (robust to non-proportional hazards), which yielded a P-value of 0.005. Fleming-Harrington weighted log-rank tests were also performed to assess early-stage ($P=0.008$) and late-stage ($P=0.74$) differences. (C) MAGI3 mRNA expression in clinical samples ($n=30$) was measured by RT-qPCR assay. (D) MAGI3 mRNA expression decreases with the increase of tumor sizes (≥ 2 cm), advancing TNM stage and lymph node metastasis. (E) MAGI3 protein expression in clinical samples ($n=30$) was measured by immunohistochemistry assay. (F and G) The mRNA and protein levels of MAGI3 in NSCLC cell lines (A549, PC9, H1155, SW900, H460, H1975, H1650, H23 and H1299) and human normal lung cell line (Beas-2b) were revealed by RT-qPCR and western blotting assay ($n=3$). ** $P < 0.01$ and *** $P < 0.001$. Data were displayed as the mean $\pm$ SD of three independent experiments. MAGI3, membrane associated guanylate kinase WW and PDZ domain containing 3; NSCLC, non-small cell lung cancer; TCGA, The Cancer Genome Atlas; RT-qPCR, reverse transcription-quantitative PCR; LUAD, lung adenocarcinoma.

was lower than of Control group. Besides, TCGA database showed that lower expression of MAGI3 is closely relevant to bad prognosis in patients with NSCLC ($P=0.023$, Fig. 1B). In clinical samples, it was also verified that the MAGI3 expression

was downregulated in NSCLC tumor tissues ($n=30$) (Fig. 1C). Additionally, the association between MAGI3 expression and tumor size, tumor-node-metastasis (TNM) stage as well as lymph node (LN) metastasis, was quantified. As shown in

Fig. 1D, the mRNA level of MAGI3 in tumor sizes (≥ 2 cm) was significantly decreased compared with tumor sizes (< 2 cm) ($P < 0.05$). MAGI3 mRNA levels decreased significantly with advancing TNM stage: stage-III and IV ($P < 0.01$). Consistently, higher rate of lymph-node metastasis cases was correlated inversely with low MAGI3 expression ($P < 0.05$). Collectively, these data quantitatively link reduced MAGI3 expression to both advanced tumor stage and increased metastasis in NSCLC. The results of IHC in the collected NSCLC pathological samples revealed that the protein expression level of MAGI3 in tumor tissues was significantly decreased compared with that in adjacent tissues ($P < 0.01$, Fig. 1E). Fisher's exact test revealed that low MAGI3 expression in NSCLC showed a trend toward association with lymph node involvement and advanced pathological tumor-node-metastasis stage, although the P-values were of borderline significance ($P = 0.0502$ for both; Table I). No significant association was observed with sex, age, or tumor size ($P > 0.05$; Table I). Notably, the mRNA expression analysis (Fig. 1D) independently confirmed that MAGI3 mRNA levels were significantly decreased in patients with advanced TNM stage (III/IV, $P < 0.01$) and lymph node metastasis ($P < 0.05$), supporting the clinical relevance of MAGI3 downregulation in NSCLC progression. Consistently, the mRNA and protein levels of MAGI3 in NSCLC cell lines were significantly decreased by comparison with human normal lung cell line (Beas-2b) ($P < 0.001$) (Fig. 1F and G).

Downregulation of MAGI3 accelerates the tumorigenesis and metastasis in the KrasLSL-G12D model. The overview of MAGI3 and KrasLSL-G12D transgenes was shown in Fig. 2A. Next, the transfection efficacy was detected and it was identified that MAGI3 expression in the lung of sg-MAGI3 group was remarkably lower than sg-GFP group ($P < 0.01$ and $P < 0.001$) (Fig. 2B-D), indicating that MAGI3 was effectively knocked down by ~ 70 -75% in mouse lungs, with residual expression attributable to the inherent heterogeneity of somatic CRISPR/Cas9 editing via intratracheal lentiviral delivery. Additionally, tumors in sg-MAGI3 group were characterized by severe pleomorphic nuclei, and high extent of desmoplastic stroma in contrast to sg-GFP group, quantitative analysis of the lung sections revealed that sgMAGI3 mice exhibited a significantly higher tumor burden (% tumor area/total lung area) compared with sgGFP controls ($P < 0.001$) (Fig. 2E). At 15 weeks, most of KrasLSL-G12D mice developed LN metastases ($P < 0.01$) (Fig. 2F). Hence, sg-MAGI3 supply a powerful role on tumorigenesis and metastasis of adenocarcinoma.

MAGI3 functions as a tumor suppressor for NSCLC cells in vitro. To further investigate the mechanisms of MAGI3 in NSCLC progression, DOX inducible Tet-on system was used *in vitro* that switches on the MAGI3 expression. Based on the results of Fig. 1F and G, the A549 and PC9 cell lines exhibit moderate or low expression of endogenous MAGI3, making them ideal models for functional acquisition (overexpression) studies and facilitating the observation of phenotypic changes after overexpression of MAGI3. At first, the efficiency of Tet-on system was validated by the MAGI3 expression in Tet-On-inducible A549 and PC9 cells after treatment with DOX. Results displayed that MAGI3 level in the DOX⁺ tet-MAGI3 group were increased in contrast to DOX⁻ tet-NC,

DOX⁺ tet-NC and DOX⁻ tet-MAGI3 groups; DOX-induced MAGI3 overexpression resulted in an ~ 1.6 -fold increase in A549 cells and 1.5-fold increase in PC9 cells compared with the non-induced controls ($P < 0.001$) (Fig. 3A). Moreover, a decrease in cell viability was observed 48 h after DOX-induced MAGI3 overexpression (Fig. 3B). In line, overexpression of MAGI3 induced by DOX inhibited the clonogenicity of A549 and PC9 cells (Fig. 3C). Sphere formation assay displayed that the numbers of spheres were lower in the DOX⁺ tet-MAGI3 group by comparison with DOX⁻ tet-NC, DOX⁺ tet-NC and DOX⁻ tet-MAGI3 groups ($P < 0.01$) (Fig. 3D). Transwell assay also presented that the cells overexpressing MAGI3 had reduced abilities of migration and invasion (Fig. 3E and F). The aforementioned evidence indicated that MAGI3 functions as a tumor suppressor in NSCLC development.

Overexpression of MAGI3 accelerates cell cycle arrest and cell senescence in NSCLC. Next, the cell cycle distribution was investigated in A549 and PC9 cells. Flow cytometry demonstrated that DOX-induced MAGI3 expression increased the percentage of cells in G1 phase, while decreased the percentage of S phase cells ($P < 0.01$) (Fig. 4A). Similarly, it was also observed that cyclinD1, as a cell cycle-related protein acting on G1-S phase transition, was obviously reduced in the DOX⁺ tet-MAGI3 group, compared with DOX⁻ tet-NC, DOX⁺ tet-NC and DOX⁻ tet-MAGI3 groups; meanwhile, parallel immunoblotting also confirmed that MAGI3 expression was successfully induced upon DOX treatment ($P < 0.001$) (Fig. 4B). Because cell cycle arrest is termed to be important feature of cellular senescence, the functions of MAGI3 overexpression on cellular senescence was further explored. As expected, the proportion of senescent cells was considerably higher in DOX induced MAGI3-overexpressing A549 and PC9 cells (Fig. 4C). Those evidence suggested the crux role of MAGI3 in inducing the cell cycle arrest and cell senescence.

Overexpression of MAGI3 restrains the Wnt pathway to promotes cell senescence of NSCLC cells. It is well established that Wnt pathway involves in the cancer processes including cell senescence (17). Therefore, the Wnt pathway-associated downstream genes were firstly detected. As presented in Fig. 5A, concurrent detection of MAGI3 confirmed its DOX-dependent induction in the same samples, establishing a direct correlation between MAGI3 upregulation and suppression of these Wnt downstream effectors. The protein levels of cMYC, c-JUN, WISP1 and PPARD in the DOX⁺ tet-MAGI3 group were lower than DOX-tet-NC, DOX⁺ tet-NC and DOX⁻ tet-MAGI3 groups ($P < 0.001$), indicating that overexpression of MAGI3 significantly restrained the Wnt pathway. To further confirm whether MAGI3 promotes cell senescence by inhibiting the Wnt pathway in NSCLC cells, Wnt agonist (HY-114321) was added into the DOX⁺ inducible MAGI3-overexpressing cells. As shown in Fig. 5B, MAGI3 overexpression significantly upregulated p16 and p21, two canonical effectors of cellular senescence. To further confirm the activation of the p53 pathway, phosphorylation of p53 at Ser15 (p-p53), a well-established functional activation marker, was monitored. As presented in Fig. S1, p-p53 levels were significantly increased upon MAGI3 overexpression, while total p53 protein levels remained unchanged across all groups, confirming that the

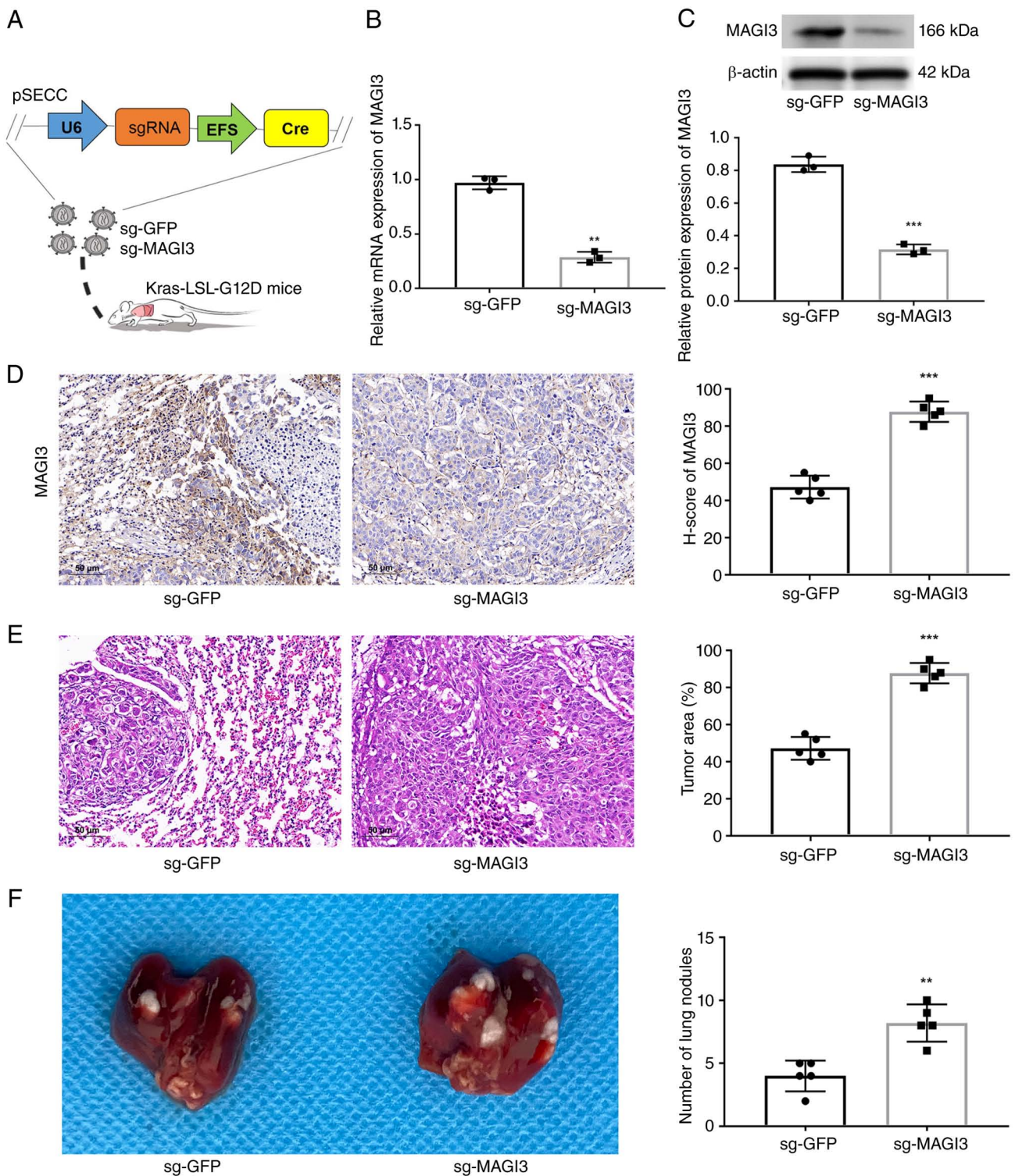


Figure 2. Downregulation of MAGI3 accelerated the lung adenocarcinoma progression in the KrasLSL-G12D model. (A) The overview of MAGI3 and KrasLSL-G12D transgenes was shown. (B-D) Reverse transcription-quantitative PCR, western blotting and immunohistochemistry assays (scale bar, 50 μ m) were utilized to detect the transfection efficacy after infection with lentiviruses containing sg-MAGI3-pSECC (n=3 independent experimental replicates). (E) H&E staining of lung sections was shown at 8 weeks after tumor initiation (scale bar, 50 μ m; n=5). (F) Representative images presented the lymph node metastases at 15 weeks; **P<0.01 and ***P<0.001. Data are displayed as the mean $\pm$ SD of KrasLSL-G12D mice (n=5). MAGI3, membrane associated guanylate kinase WW and PDZ domain containing 3.

observed phosphorylation reflects specific pathway activation rather than changes in total p53 expression. Results presented that cell senescence-associated markers including p16, p21 and p-p53 in the DOX⁺ tet-MAGI3⁺ HY-114321 group were significantly decreased in contrast to DOX⁺ tet-MAGI3 group, while

total p53 levels remained stable across groups, determining that HY-114321 can reverse the increases of cell senescence caused by overexpression of MAGI3 (P<0.001). Of note, the MAGI3 levels in the DOX⁺ tet-MAGI3⁺ HY-114321 cohort were comparable to those in the DOX⁺ tet-MAGI3 group,

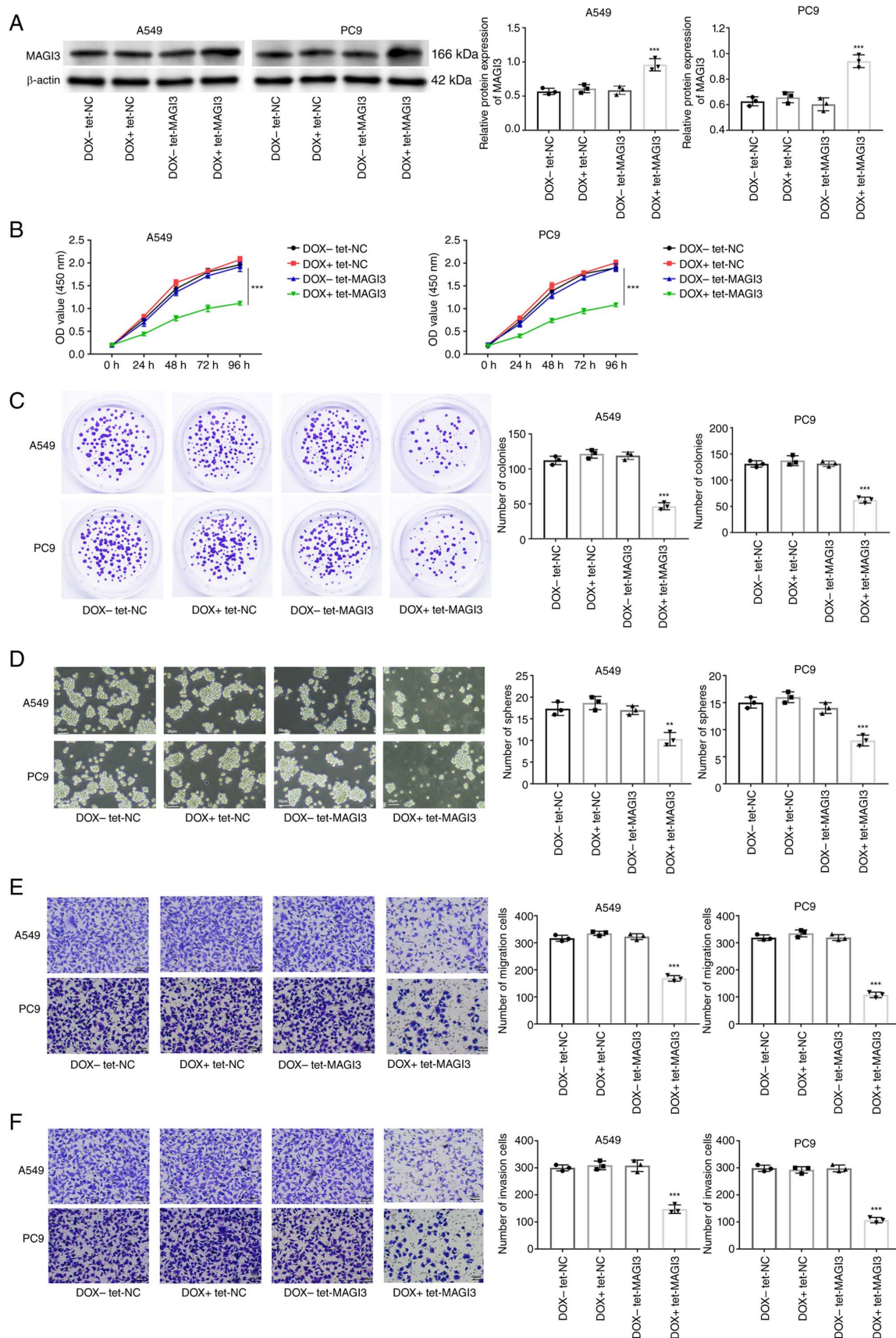


Figure 3. MAGI3 functions as a tumor suppressor for non-small cell lung cancer cells *in vitro*. (A) The efficiency of Tet-on system inducible MAGI3 overexpression was determined by western blotting. (B) Cell Counting Kit-8 was employed to detect the cell viability in MAGI3-overexpressing cells. (C) The influences of Tet-on system inducible MAGI3 overexpression on clonogenicity in A549 and PC9 cells was evaluated by colony formation assay. (D) Sphere formation assay was adopted to display that the numbers of spheres in DOX-induced MAGI3-overexpressing cells (scale bar, 50 μ m). (E and F) The influences of Tet-on system inducible MAGI3 overexpression on migration and invasion was tested by Transwell assay (scale bar, 100 μ m). ** $P < 0.01$ and *** $P < 0.001$; Data were displayed as the mean $\pm$ SD of three independent experiments. MAGI3, membrane associated guanylate kinase WW and PDZ domain containing 3; DOX, doxycycline; NC, negative control.

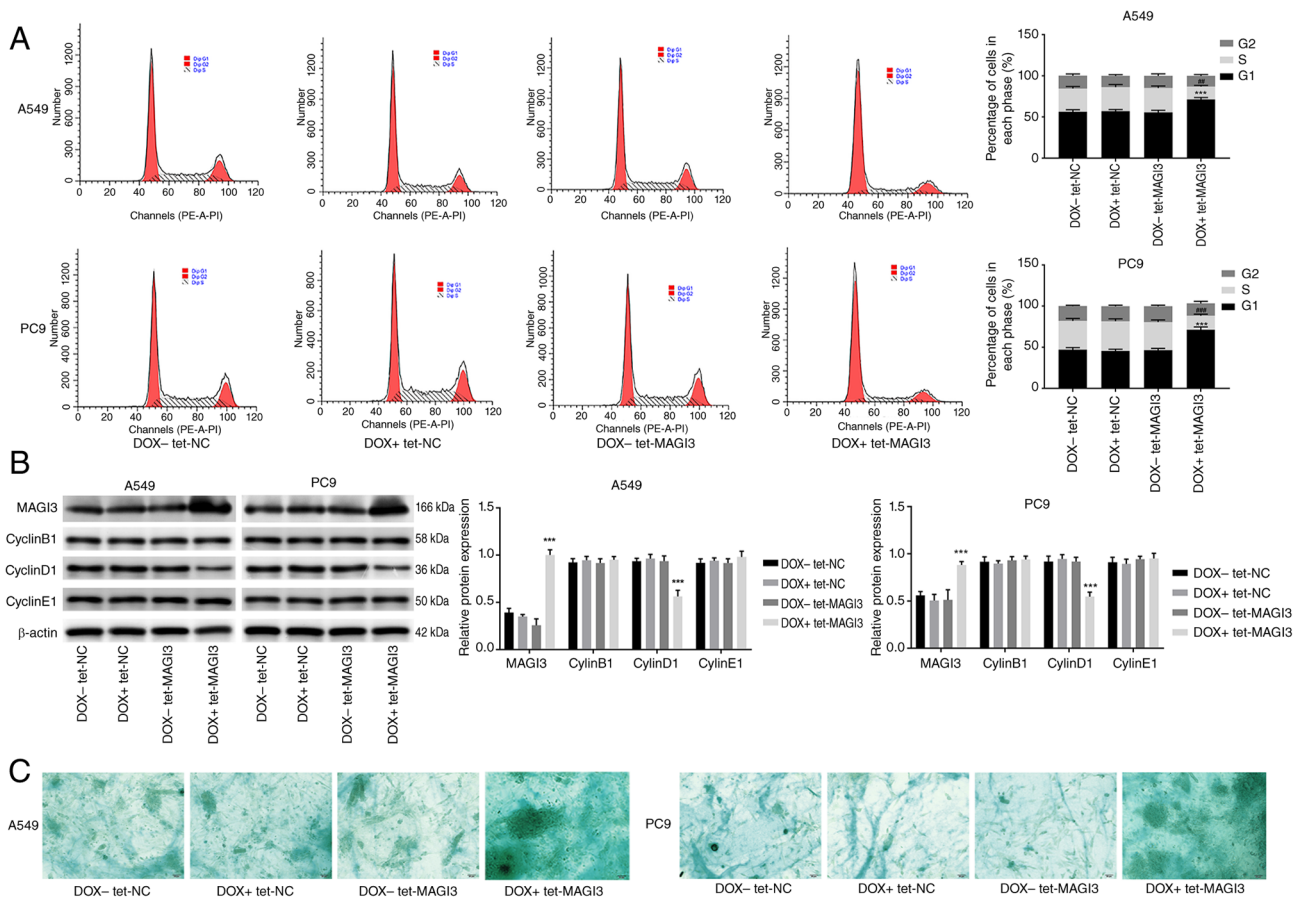


Figure 4. Overexpression of MAGI3 accelerates cell cycle arrest and cell senescence in non-small cell lung cancer. (A) The impacts of Tet-on system inducible MAGI3 overexpression on cell cycle was evaluated by flow cytometry. (B) Western blotting was employed to test the levels of cyclinB1, cyclinD1 and cyclinE1 in DOX-induced MAGI3-overexpressing cells. (C) The functions of MAGI3 overexpression on cellular senescence was explored by β -Galactosidase staining assay (scale bar, 20 μ m). *** P <0.001. ** P <0.01 and # P <0.001. Data were displayed as the mean $\pm$ SD of three independent experiments. MAGI3, membrane associated guanylate kinase WW and PDZ domain containing 3; DOX, doxycycline; NC, negative control.

verifying that HY-114321 did not affect DOX-induced MAGI3 upregulation (Figs. 5B and S1). To assess whether MAGI3 influences β -catenin distribution, fractionation assays were conducted in DOX-inducible MAGI3-overexpressing A549 cells. As shown in Fig. 5C, MAGI3 overexpression markedly lowered nuclear β -catenin protein, whereas its cytoplasmic counterpart stayed relatively stable. This suggests that MAGI3 primarily hinders β -catenin nuclear translocation, rather than altering its total protein content. The effect of MAGI3 on other Wnt pathway constituents was next explored. As presented in Fig. 5D, MAGI3 overexpression significantly decreased the protein level of DVL2, a critical adaptor in Wnt signal transduction. Additionally, the phosphorylation of GSK3 β at Ser9 (p-GSK3 β), which is indicative of Wnt pathway activation, was significantly reduced upon MAGI3 overexpression (P <0.001), whereas total GSK3 β protein levels remained unchanged. These results suggest that MAGI3 suppresses Wnt signaling at multiple levels, including reduction of DVL2 and inhibition of GSK3 β inactivation (P <0.001). To directly evaluate the transcriptional activity of the Wnt/ β -catenin pathway, TOP/FOP Flash luciferase reporter assays were conducted. As illustrated in Fig. 5E, DOX-induced MAGI3 overexpression significantly reduced TOP-Flash luciferase activity compared with control groups (P <0.001), while FOP-Flash activity showed no significant changes. These findings provide direct functional evidence

that MAGI3 suppresses TCF/LEF-mediated transcriptional activation, further supporting the conclusion that MAGI3 inhibits the Wnt pathway in NSCLC cells. Collectively, it is considered that overexpression of MAGI3 can restrain Wnt pathway to boost cell senescence in NSCLC cells.

MAGI3 suppresses the Wnt pathway by degradation of TAZ. MAGI3 is a PDZ-protein, and TAZ contains a PDZ-binding motif. As a result, there is possibility of MAGI3 interacting with TAZ (18). First, the data showed that TAZ level in the DOX⁺ tet-MAGI3 group was significantly decreased in contrast to DOX⁺ tet-NC, DOX⁻ tet-NC and DOX⁻ tet-MAGI3 groups (P <0.001); and MAGI3 immunoblotting in parallel confirmed that this TAZ reduction was dependent on DOX-induced MAGI3 expression (Fig. 6A). Co-IP confirmed that MAGI3 bound TAZ in 293T and A549 cells (Fig. 6B and C). Beyond that, DOX⁺ inducible MAGI3 overexpression in A549 cells outstandingly reduced the half-life of TAZ compared with tet-MAGI3 A549 cells without DOX treatment (Fig. 6D). Considering that ubiquitination of TAZ induces its degradation, it was hypothesized that MAGI3 might target TAZ protein degradation through ubiquitination of TAZ. It was revealed that TAZ ubiquitination was higher in the DOX⁺ tet-MAGI3 group than those of DOX⁻ tet-MAGI3 group (Fig. 6E). To determine whether the MAGI3-TAZ interaction

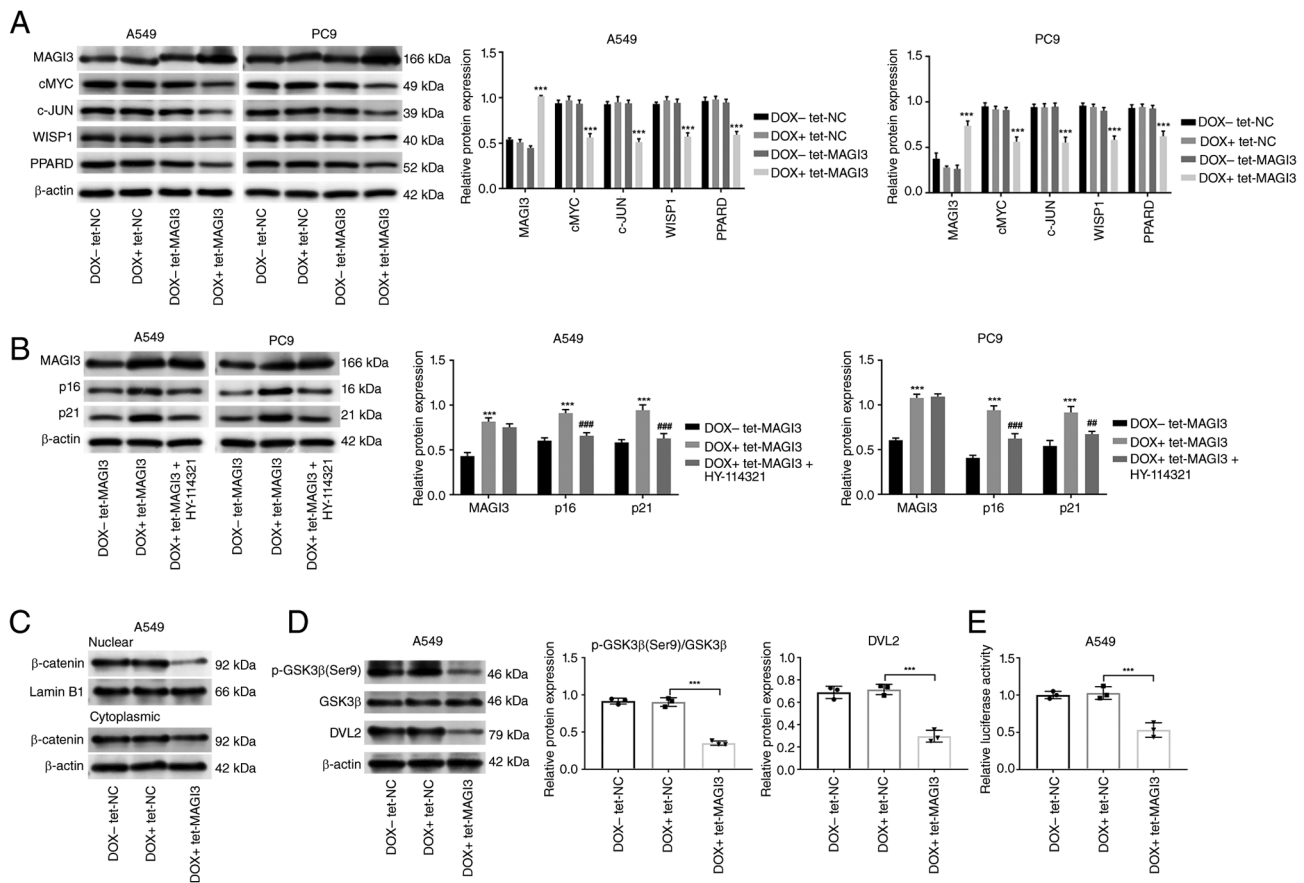


Figure 5. Overexpression of MAGI3 restrains the Wnt pathway to promote cell senescence of non-small cell lung cancer cells. (A) The Wnt pathway-associated markers including cMYC, c-JUN, WISP1 and PPARD were examined by western blotting. (B) The protein levels of p16 and p21 were utilized to estimate the interaction between MAGI3 and Wnt agonist (HY-114321) on cell senescence. (C) Nuclear and cytoplasmic fractionation followed by western blotting showing β-catenin distribution in A549 cells with or without DOX-induced MAGI3 overexpression. Lamin B1 and β-actin served as nuclear and cytoplasmic markers, respectively. (D) Western blotting analysis of DVL2, p-GSK3β (Ser9) and total GSK3β in A549 cells upon MAGI3 overexpression. (E) TOP/FOP Flash luciferase reporter assay showing TCF/LEF transcriptional activity in A549 cells with DOX-inducible MAGI3 overexpression. *** $P < 0.001$ vs. DOX- tet-NC, DOX+ tet-NC and DOX- tet-MAGI3 groups; ### $P < 0.01$ and ### $P < 0.001$ vs. DOX+ tet-MAGI3 group; Data were displayed as the mean $\pm$ SD of three independent experiments. MAGI3, membrane associated guanylate kinase WW and PDZ domain containing 3; DOX, doxycycline; p-, phosphorylated; NC, negative control.

depends on the canonical PDZ domain-PDZ-binding motif recognition, a TAZ mutant with a disrupted PDZ-binding motif (TAZ-ΔPDZBM, with the C-terminal SSWV motif mutated to SSAA) was generated. Co-IP assays revealed that MAGI3 robustly bound to wild-type TAZ, whereas this interaction was markedly diminished with TAZ-ΔPDZBM (Fig. 6F), indicating that the PDZ-binding motif of TAZ is essential for MAGI3 binding. Consistently, when the PDZ domain of MAGI3 was deleted (MAGI3-ΔPDZ), its association with TAZ was also substantially impaired (Fig. 6G). It was next examined whether this PDZ-dependent interaction is functionally required for MAGI3-mediated TAZ ubiquitination. As shown in Fig. 6H, MAGI3 overexpression strongly promoted ubiquitination of wild-type TAZ. However, ubiquitination of TAZ-ΔPDZBM was barely detectable even in the presence of MAGI3 overexpression, demonstrating that the PDZ-binding motif of TAZ is indispensable for MAGI3-induced ubiquitination and subsequent degradation. Collectively, these data confirm that MAGI3 specifically recognizes TAZ through PDZ domain-mediated interaction, and this recognition is a prerequisite for TAZ ubiquitination. Moreover, it was found that the Wnt pathway-related markers including cMYC, c-JUN, WISP1 and PPARD in the DOX+ tet-MAGI3 + TAZ group were higher

than those of DOX+ tet-MAGI3 group ($P < 0.001$); and MAGI3 blots confirmed equivalent DOX-induced MAGI3 expression across all groups, ensuring the rescue effects were specifically attributable to TAZ reconstitution (Fig. 6I), indicating that TAZ can reverse the decreases of Wnt pathway-related markers caused by MAGI3 overexpression. Thus, it was considered that MAGI3 suppressed the Wnt pathway by degradation of TAZ. Additionally, it was also observed that overexpression of TAZ could reverse the decline in cell viability of NSCLC caused by MAGI3 ($P < 0.001$) (Fig. 6J). To validate the physiological relevance of MAGI3 as a tumor suppressor, loss-of-function experiments were performed in A549 cells using shRNA against MAGI3 and siRNA against TAZ ($P < 0.001$; Fig. S2A). shMAGI3 reduced MAGI3 and elevated TAZ, confirming negative regulation; co-transfection with siTAZ reversed TAZ accumulation to below baseline. MAGI3 knockdown enhanced proliferation (CCK-8), colony formation, migration and invasion (all $P < 0.001$; Fig. S2B-E). Concurrent TAZ knockdown abrogated these effects, whereas TAZ knockdown alone suppressed these phenotypes. Thus, MAGI3 loss-driven oncogenic effects depend largely on TAZ accumulation. At the molecular level, MAGI3 depletion upregulated Wnt effectors cMYC, c-JUN, WISP1 and PPARD ($P < 0.001$; Fig. S2F),

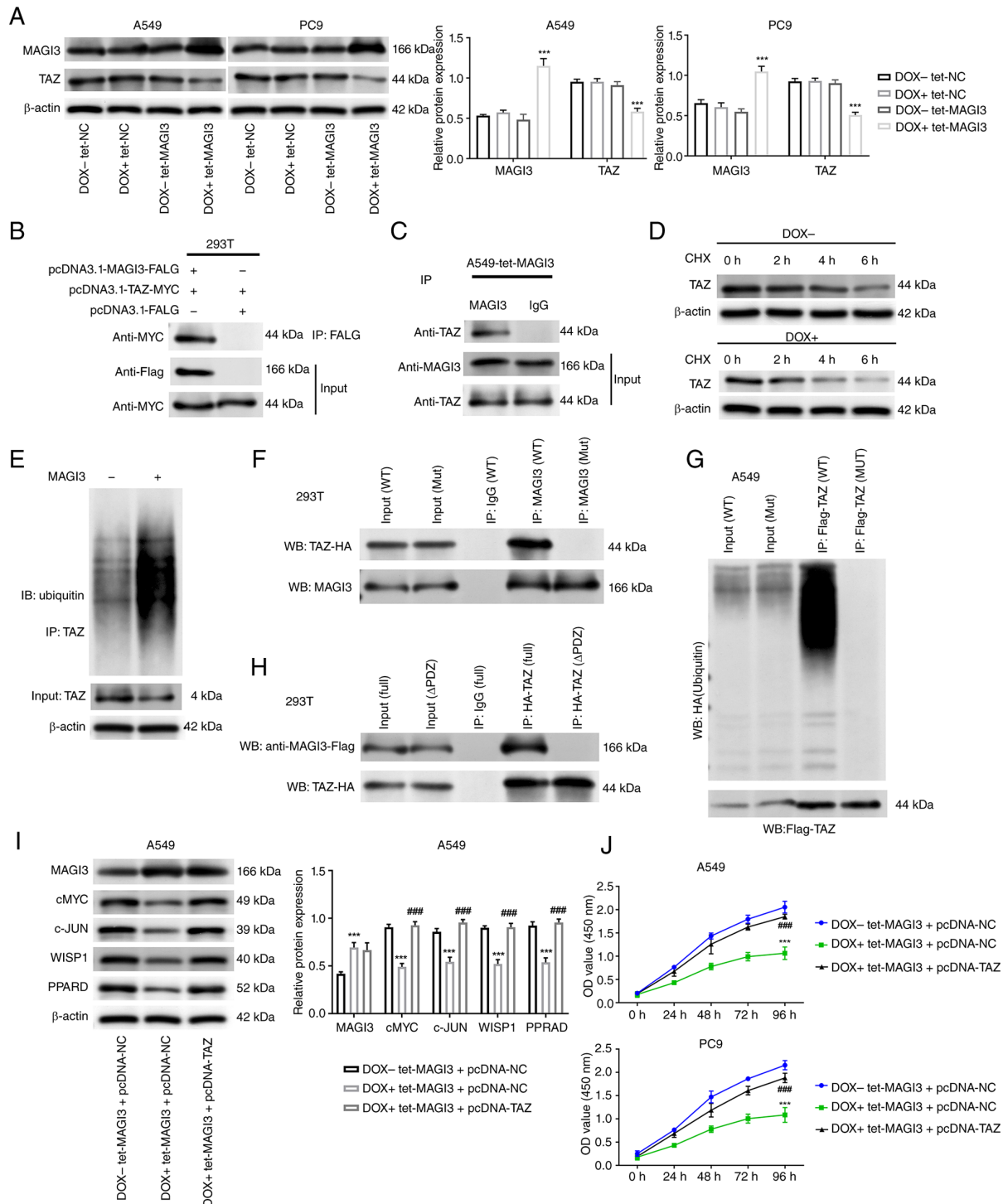


Figure 6. MAGI3 suppresses the Wnt pathway by degradation of TAZ. (A) The impact of MAGI3 overexpression on TAZ level was monitored by western blotting. (B and C) The relation between MAGI3 and STZ in 293T and A549 cells was confirmed by Co-IP assay. (D) Half-Life detection expressed that DOX inducible MAGI3 overexpression in A549 cells outstandingly reduced the TAZ stability. (E) Ubiquitination assay presented that MAGI3 boosted the TAZ ubiquitination to induce degradation. (F) Co-IP assay showing that mutation of the PDZ-binding motif in TAZ (TAZ- Δ PDZBM) abolished its interaction with MAGI3. (G) Co-IP assay showing that deletion of the PDZ domain in MAGI3 (MAGI3- Δ PDZ) impaired its binding to TAZ. (H) Ubiquitination assay demonstrating that TAZ- Δ PDZBM was resistant to MAGI3-induced ubiquitination. (I) The interaction between MAGI3 and TAZ on Wnt pathway-related markers was tested by western blotting. (J) Cell Counting Kit-8 was employed to detect the cell viability in MAGI3 and TAZ-overexpressing cells. ***P<0.001 vs. DOX- tet-NC, DOX+ tet-NC and DOX- tet-MAGI3 groups; **P<0.01 vs. DOX+ tet-MAGI3 + pcDNA-NC group; Data are displayed as the mean $\pm$ SD of three independent experiments. MAGI3, membrane associated guanylate kinase WW and PDZ domain containing 3; Co-IP, Co-immunoprecipitation; DOX, doxycycline; WT, wild-type; Mut, mutant; NC, negative control.

indicating pathway activation. This upregulation was reversed by TAZ co-knockdown, corroborating that MAGI3 represses Wnt signaling in a TAZ-dependent manner.

MAGI3 links the TAZ by which suppresses the NSCLC metastasis via Wnt pathway in vivo. To further verify whether MAGI3 impacts an oncogenic role *in vivo*, xenograft

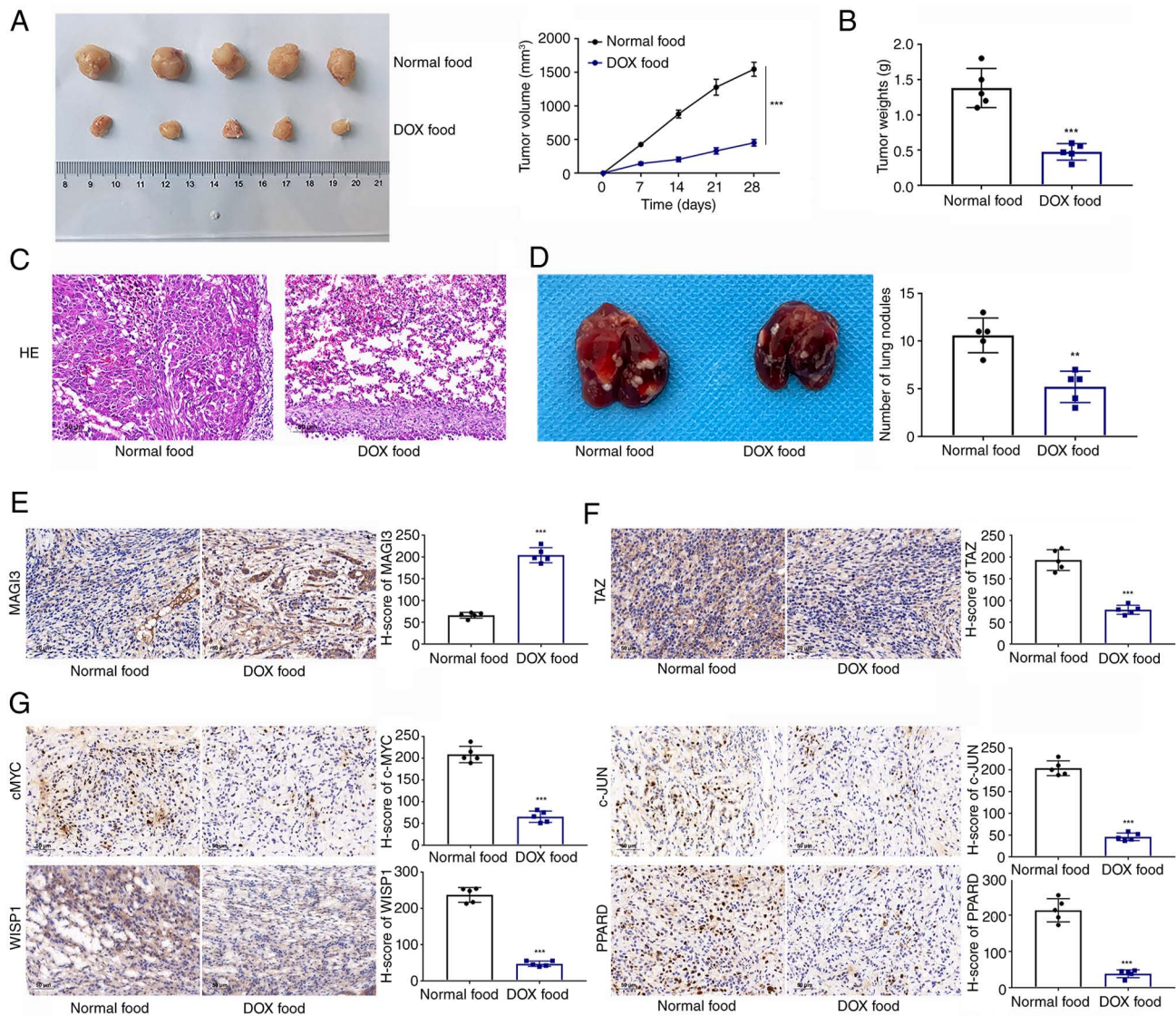


Figure 7. MAGI3 links the TAZ by which suppresses the non-small cell lung cancer metastasis via Wnt pathway in xenograft tumor model. (A) Representative images of tumors and tumor volumes in each group (n=5 mice). (B) The role of MAGI3 on tumor weight was displayed. (C) H&E staining of lung sections was shown at 8 weeks after tumor initiation (scale bar, 50 μ m). (D) Representative images presented the inhibitory effects of DOX-induced MAGI3-overexpression on lung metastasis (scale bar, 50 μ m); (E-G) Immunohistochemistry assay was adopted to test the TAZ, cMYC, c-JUN, WISP1 and PPARD expressions in DOX-induced MAGI3-overexpressing tumors (scale bar, 50 μ m); *P<0.01 and ***P<0.001 vs. normal food. Data were displayed as the mean $\pm$ SD of BALB/c-nu/nu mice (n=5). MAGI3, membrane associated guanylate kinase WW and PDZ domain containing 3; DOX, doxycycline.

tumor model was employed and data displayed that the MAGI3-expressing cells in presence of DOX led to a prominently decreased tumor volumes and tumor weights, compared with absence of DOX in the MAGI3-expressing cells (Fig. 7A and B). H&E staining also confirmed the inhibitory effects of DOX-induced MAGI3-overexpression on lung metastasis (Fig. 7C). Furthermore, the roles of MAGI3 on tumor metastatic capacity were investigated *in vivo*, and it was found that DOX-induced MAGI3-overexpression significantly decreased the number of metastatic lung nodules (P<0.01; Fig. 7D). Moreover, IHC assay presented the reduction of TAZ, cMYC, c-JUN, WISP1 and PPARD in DOX-induced MAGI3-overexpressing tumors (P<0.001; Fig. 7E-G). Taken together, the present data certified that DOX-induced MAGI3 overexpression can link the TAZ to modulate Wnt pathway, by which mediates tumor growth and metastasis *in vivo*.

Discussion

An in-depth understanding of the underlying mechanisms and functions of E3 ubiquitin ligase in tumorigenesis is expected to facilitate the development of anticancer therapies (19). In the present study, the *in vitro* findings together with mouse model, highlighted MAGI3 as a tumor suppressor gene in NSCLC progression. Additionally, it was also demonstrated that MAGI3 degrades the TAZ protein via its ubiquitination to restrain the Wnt pathway in NSCLC cells.

It is intriguing that mutation and dysregulation of MAGI3 family is frequent in cancer progression (20,21). MAGI3, belongs to the subgroup of membrane-associated guanylate kinases (MAGUK), which have been identified as a hub gene in lung cancer (22). Similarly, MAGI2, a considerable homologue, also exerts the crux impacts on advanced lung

adenocarcinoma (23). The present study revealed that MAGI3 was downregulated and relevant to prognosis in NSCLC. Besides, DOX⁺ inducible MAGI3 overexpression restrained the clonogenicity, sphere formation, migration and invasion, and accelerated cell cycle arrest and cell senescence, thus influencing the tumorigenesis and metastasis. *In vivo* study presented that MAGI3 overexpression suppressed tumor growth and LN metastases as well. Those findings supported the previous evidence that MAGI3 functions as a tumor suppressor in human tumors (24-26). Importantly, this tumor-suppressive role was further corroborated by loss-of-function rescue experiments, wherein MAGI3 knock-down promoted malignant phenotypes whereas concurrent TAZ depletion effectively reversed these effects, establishing a bidirectional causal link between MAGI3 loss and NSCLC aggressiveness.

Due to the structural feature of MAGI3 containing PDZ domains, WW domains and GUK domain, it can target a variety of proteins and further modulate their-related signaling pathways (20,27). In cancer progression, several studies have clarified the functional association of MAGI3 and Wnt pathway. For instance, Katoh (11) has verified that MAGI3 integrates with substrate molecules to assemble Wnt/PCP signaling molecules. Additionally, Ma *et al* (25) have also confirmed that MAGI3 restrains glioma malignant phenotype via negatively mediating the Wnt pathway. In line, current data presented that overexpression of MAGI3 significantly restrained the Wnt pathway, determined by the protein levels of cMYC, c-JUN, WISP1 and PPAR δ in the DOX⁺ tet-MAGI3 group were lower than DOX-tet-NC, DOX⁺ tet-NC and DOX⁻ tet-MAGI3 groups. Furthermore, it was also found that Wnt agonist (HY-114321) can reverse the increases of cell senescence caused by overexpression of MAGI3. Correspondingly, previous studies have explained that inhibition of Wnt signaling induces cellular senescence, followed by decreasing cancer stemness (28,29). Thus, this evidence manifested the functional mechanism of MAGI3 on Wnt pathway in malignant phenotype of NSCLC cells.

Although both prior findings and the present data testified that MAGI3 suppressed tumor progression by reducing Wnt pathway-related markers, the exact molecular mechanism by how MAGI3 influences Wnt pathway still requires further exploration. In the current study, it was found that MAGI3 overexpression reduced TAZ stability by accelerating the ubiquitination of TAZ protein to induce degradation. Consistently, Yuan *et al* (30) have presented evidence that destabilizing TAZ can notably refrain the osteosarcoma progression. In regulation of lung cancer, YAP/TAZ stabilization exerts the function in tumor stemness, which has been testified (31). Those evidence indicated that TAZ stability is a key for NSCLC development. The ubiquitin-proteasome system is the dominating modulator of protein degradation, and aberrant of this system can eventually influence the tumorigenesis (32). MAGI3, is an E3 ubiquitin ligase containing PDZ domain that regulates various aspects of eukaryotes by facilitating ubiquitination and degradation of proteins (33). Besides, a previous study uncovered the TAZ structure and clarified TAZ containing a PDZ-binding motif, demonstrating that TAZ acts as a transcriptional co-activator modulated by coaction with PDZ domain proteins (18). Thus, it is considered that the PDZ domain of MAGI3 can bind

TAZ and subsequently modulate TAZ ubiquitination and degradation. While a comprehensive substrate scan was not performed, the combination of MAGI3-induced ubiquitination detected only for TAZ and exclusive functional rescue by TAZ indicates that, among the PDZ-binding proteins examined, TAZ is the preferential substrate of MAGI3 in NSCLC cells. This notion is further substantiated by the domain-mapping experiments of the present study. It was demonstrated that mutation of the PDZ-binding motif in TAZ (TAZ- Δ PDZBM, mutating the C-terminal SSWV to SSAA) or deletion of the PDZ domain in MAGI3 (MAGI3- Δ PDZ) markedly abrogated their interaction. Critically, TAZ- Δ PDZBM was resistant to MAGI3-induced ubiquitination, confirming that the PDZ domain-mediated recognition is the primary determinant conferring substrate specificity in this regulatory axis.

On other hand, TAZ, as an oncoprotein, is involved in the several cellular processes including cellular senescence (34), DNA repair response (35), migration and invasion (36). Additionally, other studies have verified that TAZ stability is required for Wnt pathway-related gene expression in tumorigenesis (9,37,38). Based on the aforementioned findings, the present study provides the key evidence that MAGI3 overexpression can link the TAZ and then functional crosstalk with Wnt pathway in NSCLC. Collectively, the feedback loop E3 ligase MAGI3-mediated TAZ ubiquitination and then targeting the Wnt pathway is vital for tumor growth and metastasis of NSCLC.

Despite the comprehensive *in vitro* and *in vivo* evidence demonstrating the tumor-suppressive role of MAGI3 and its regulation of the TAZ/Wnt axis, several limitations should be acknowledged. First, it is acknowledged that the *in vivo* KrasLSL-G12D/sgMAGI3 data reflect partial MAGI3 knock-down (~70-75% efficiency) rather than complete ablation, owing to the inherent heterogeneity of somatic CRISPR/Cas9 editing. Although this partial depletion was biologically sufficient to drive significant tumor phenotypes and aligned with the present *in vitro* rescue data, an independent sgRNA was not pursued for validation. This decision was made primarily to adhere to the 3R principles and the approved animal protocol. Therefore, the *in vivo* conclusions should be interpreted within the context of partial depletion; future studies using additional sgRNAs or conditional knockout models would further consolidate these findings. Second, although domain-mapping and mutagenesis data establish PDZ-dependent TAZ ubiquitination by MAGI3 in cells, *in vitro* validation with purified proteins was not performed, owing to the technical difficulty of expressing full-length MAGI3 in prokaryotic systems and its probable reliance on mammalian-specific modifications or partners. Nevertheless, the cell-based rescue and mutagenesis experiments strongly support direct ubiquitination, and future biochemical studies could further elucidate the catalytic mechanism. Third, TAZ-independent tumor-suppressive functions of MAGI3 cannot be excluded. Fourth, the upstream mechanisms underlying MAGI3 downregulation in NSCLC, such as promoter hypermethylation, genomic deletion, or microRNAs, remain to be clarified. Fifth, it is acknowledged that, due to ethical restrictions and tissue availability in the KrasLSL-G12D model (limited to H&E and MAGI3 IHC per approved protocol), TAZ and Wnt marker expression were not examined in these specific lung tissues. Although

this represents an unaddressed limitation at the tissue level in this autochthonous model, the *in vivo* relevance is strongly supported by xenograft molecular data (Fig. 7) and comprehensive *in vitro* rescue experiments. It is explicitly noted that direct confirmation in the Kras model would be desirable in future studies. Finally, the mechanistic findings of the present study are primarily derived from established cell lines; validation in larger clinical cohorts and primary tumor samples is essential, especially given the modest sample sizes (n=30 patients; n=5 per group), which warrant cautious interpretation.

In conclusion, the present study identified MAGI3 as a suppressor in NSCLC progression. Importantly, the mechanism by which MAGI3, an E3 ligase, promotes TAZ ubiquitination and degradation, was also revealed, which correlates with suppression of the Wnt pathway, thereby restraining tumorigenesis and metastasis of NSCLC. These data may offer the clues to boost understanding for pathogenesis of NSCLC.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YL and JZ conceptualized the study, curated data, conducted formal analysis, wrote the original draft and wrote, reviewed and edited the manuscript. All authors read and approved the final version of the manuscript. YL and JZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Qingdao Integrated Chinese and Western Medicine Hospital (approval no. 202528; Qingdao, China) and conducted in consistent with the declaration of Helsinki. Informed consent was obtained from all participants. All animal experiments were conformed to the animal care and use guidelines and approved by the Ethics Committee of Beijing Viewsolid Biotechnology Co. LTD (approval no. VS2124A0023). All the operations followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and ARRIVE Guidelines pertaining to animal experimentation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD, Fuchs HE and Jemal A: Cancer statistics, 2022. *CA Cancer J Clin* 72: 7-33, 2022.
2. Zappa C and Mousa SA: Non-small cell lung cancer: Current treatment and future advances. *Transl Lung Cancer Res* 5: 288-300, 2016.
3. Heist RS and Engelman JA: SnapShot: Non-small cell lung cancer. *Cancer Cell* 21: 448.e2, 2012.
4. Zancanato F, Cordenonsi M and Piccolo S: YAP/TAZ at the roots of cancer. *Cancer Cell* 29: 783-803, 2016.
5. Zhao C, Gong J, Bai Y, Yin T, Zhou M, Pan S, Liu Y, Gao Y, Zhang Z, Shi Y, *et al*: A self-amplifying USP14-TAZ loop drives the progression and liver metastasis of pancreatic ductal adenocarcinoma. *Cell Death Differ* 30: 1-15, 2023.
6. Gu JJ, Rouse C, Xu X, Wang J, Onaitis MW and Pendergast AM: Inactivation of ABL kinases suppresses non-small cell lung cancer metastasis. *JCI Insight* 1: e89647, 2016.
7. Lustig B and Behrens J: The Wnt signaling pathway and its role in tumor development. *J Cancer Res Clin Oncol* 129: 199-221, 2003.
8. Stewart DJ: Wnt signaling pathway in non-small cell lung cancer. *J Natl Cancer Inst* 106: djt356, 2014.
9. Azzolin L, Zancanato F, Bresolin S, Forcato M, Basso G, Biciatto S, Cordenonsi M and Piccolo S: Role of TAZ as mediator of wnt signaling. *Cell* 151: 1443-1456, 2012.
10. Fagerberg L, Hallström BM, Oksvold P, Kampf C, Djureinovic D, Odeberg J, Habuka M, Tahmasebpour S, Danielsson A, Edlund K, *et al*: Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. *Mol Cell Proteomics* 13: 397-406, 2014.
11. Katoh M: WNT/PCP signaling pathway and human cancer (review). *Oncol Rep* 14: 1583-1588, 2005.
12. Wang H, Yang W, Qin Q, Yang X, Yang Y, Liu H, Lu W, Gu S, Cao X, Feng D, *et al*: E3 ubiquitin ligase MAGI3 degrades c-Myc and acts as a predictor for chemotherapy response in colorectal cancer. *Mol Cancer* 21: 151, 2022.
13. Hiyama Y, Yamaoka E, Fukazawa T, Kojima M, Sotomaru Y and Hiyama E: In vitro transfection of up-regulated genes identified in favorable-outcome neuroblastoma into cell lines. *Cells* 11: 3171, 2022.
14. Zhao Z, Gao J, Li C, Xu X, Hu Y and Huang S: Reactive oxygen species induce endothelial differentiation of liver cancer Stem-Like sphere cells through the activation of Akt/IKK signaling pathway. *Oxid Med Cell Longev* 2020: 1621687, 2020.
15. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
16. Sánchez-Rivera FJ, Papagiannakopoulos T, Romero R, Tammela T, Bauer MR, Bhutkar A, Joshi NS, Subbaraj L, Bronson RT, Xue W and Jacks T: Rapid modelling of cooperating genetic events in cancer through somatic genome editing. *Nature* 516: 428-431, 2014.
17. Milanovic M, Fan DNY, Belenki D, Däbritz JHM, Zhao Z, Yu Y, Dörr JR, Dimitrova L, Lenze D, Monteiro Barbosa IA, *et al*: Senescence-associated reprogramming promotes cancer stemness. *Nature* 553: 96-100, 2018.
18. Kanai F, Marignani PA, Sarbassova D, Yagi R, Hall RA, Donowitz M, Hisaminato A, Fujiwara T, Ito Y, Cantley LC and Yaffe MB: TAZ: A novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins. *EMBO J* 19: 6778-6791, 2000.
19. Sévère N, Dieudonné FX and Marie PJ: E3 ubiquitin ligase-mediated regulation of bone formation and tumorigenesis. *Cell Death Dis* 4: e463, 2013.
20. Kotelevets L and Chastre E: A new story of the three magi: Scaffolding proteins and lncRNA suppressors of cancer. *Cancers (Basel)* 13: 4264, 2021.
21. Wörthmüller J and Rüegg C: MAGI1, a scaffold protein with tumor suppressive and vascular functions. *Cells* 10: 1494, 2021.
22. Wang Y, Mei Q, Ai YQ, Li RQ, Chang L, Li YF, Xia YX, Li WH and Chen Y: Identification of lung cancer oncogenes based on the mRNA expression and single nucleotide polymorphism profile data. *Neoplasma* 62: 966-973, 2015.
23. Kitamura K, Seike M, Okano T, Matsuda K, Miyanaga A, Mizutani H, Noro R, Minegishi Y, Kubota K and Gemma A: MiR-134/487b/655 cluster regulates TGF- β -induced epithelial-mesenchymal transition and drug resistance to gefitinib by targeting MAGI2 in lung adenocarcinoma cells. *Mol Cancer Ther* 13: 444-453, 2014.

24. Yang Z, Liu H, Song R, Lu W, Wang H, Gu S, Cao X, Chen Y, Liang J, Qin Q, *et al*: Reduced MAGI3 level by HPV18E6 contributes to Wnt/ β -catenin signaling activation and cervical cancer progression. *FEBS Open Bio* 11: 3051-3062, 2021.
25. Ma Q, Zhang Y, Meng R, Xie KM, Xiong Y, Lin S, He ZL, Tao T, Yang Y, Zhao JZ and He JQ: MAGI3 suppresses glioma cell proliferation via upregulation of PTEN expression. *Biomed Environ Sci* 28: 502-509, 2015.
26. Weng Q, Chen M, Yang W, Li J, Fan K, Xu M, Weng W, Lv X, Fang S, Zheng L, *et al*: Integrated analyses identify miR-34c-3p/MAGI3 axis for the Warburg metabolism in hepatocellular carcinoma. *FASEB J* 34: 5420-5434, 2020.
27. Wu Y, Dowbenko D, Spencer S, Laura R, Lee J, Gu Q and Lasky LA: Interaction of the tumor suppressor PTEN/MMAC with a PDZ domain of MAGI3, a novel membrane-associated guanylate kinase. *J Biol Chem* 275: 21477-21485, 2000.
28. Ramachandran I, Ganapathy V, Gillies E, Fonseca I, Sureban SM, Houchen CW, Reis A and Queimado L: Wnt inhibitory factor 1 suppresses cancer stemness and induces cellular senescence. *Cell Death Dis* 5: e1246, 2014.
29. Xiong J, Jiang P, Zhong L and Wang Y: The novel tumor suppressor gene ZNF24 induces THCA cells senescence by regulating wnt signaling pathway, resulting in inhibition of THCA tumorigenesis and invasion. *Front Oncol* 11: 646511, 2021.
30. Yuan P, Feng Z, Huang H, Wang G, Chen Z, Xu G, Xie Z, Jie Z, Zhao X, Ma Q, *et al*: USP1 inhibition suppresses the progression of osteosarcoma via destabilizing TAZ. *Int J Biol Sci* 18: 3122-3136, 2022.
31. Noto A, De Vitis C, Pisanu ME, Roscilli G, Ricci G, Catizone A, Sorrentino G, Chianese G, Tagliatela-Scafati O, Triscioglio D, *et al*: Stearoyl-CoA-desaturase 1 regulates lung cancer stemness via stabilization and nuclear localization of YAP/TAZ. *Oncogene* 36: 4573-4584, 2017.
32. Ciechanover A: The unravelling of the ubiquitin system. *Nat Rev Mol Cell Biol* 16: 322-324, 2015.
33. Zheng N and Shabek N: Ubiquitin ligases: Structure, function, and regulation. *Annu Rev Biochem* 86: 129-157, 2017.
34. Miyajima C, Kawarada Y, Inoue Y, Suzuki C, Mitamura K, Morishita D, Ohoka N, Imamura T and Hayash H: Transcriptional coactivator TAZ negatively regulates tumor suppressor p53 activity and cellular senescence. *Cells* 9: 171, 2020.
35. Elaimy AL, Amante JJ, Zhu LJ, Wang M, Walmsley CS, FitzGerald TJ, Goel HL and Mercurio AM: The VEGF receptor neuropilin 2 promotes homologous recombination by stimulating YAP/TAZ-mediated Rad51 expression. *Proc Natl Acad Sci USA* 116: 14174-14180, 2019.
36. Warren JSA, Xiao Y and Lamar JM: YAP/TAZ activation as a target for treating metastatic cancer. *Cancers (Basel)* 10: 115, 2018.
37. Azzolin L, Panciera T, Soligo S, Enzo E, Bicciato S, Dupont S, Bresolin S, Frasson C, Basso G, Guzzardo V, *et al*: YAP/TAZ incorporation in the β -catenin destruction complex orchestrates the Wnt response. *Cell* 158: 157-170, 2014.
38. Kim M and Jho EH: Cross-talk between Wnt/ β -catenin and Hippo signaling pathways: A brief review. *BMB Rep* 47: 540-545, 2014.



Copyright © 2026 Liang and Zhuang. This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.