Research paper

The antineoplastic drug metformin downregulates YAP by interfering with IRF-1 binding to the YAP promoter in NSCLC

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ABSTRACT

Background: Activation of the oncogene YAP has been shown to be related to lung cancer progression and associates with poor prognosis and metastasis. Metformin is a drug commonly used in the treatment of diabetes and with anticancer activity. However, the mechanism through which metformin inhibits tumorigenesis via YAP is poorly understood.

Methods: The mRNA and protein expressions were analyzed by RT-PCR and western blot. The cellular proliferation was detected by CCK8 and MTT. The cell migration and invasion growth were analyzed by wound healing assay and transwell assay. The activities of promoter were analyzed by luciferase reporter assay. Chromatin immunoprecipitation detected the combining ability of IRF-1 and 5′UTR-YAP.

Findings: Our immunohistochemistry staining and RT-PCR assays showed that the expression of YAP was higher in lung carcinoma samples. Interestingly, metformin was able to downregulate YAP mRNA and protein expression in lung cancer cells. Mechanistically, we found that metformin depressed YAP promoter by competing with the binding of the transcription factor IRF-1 in lung cancer cells. Moreover, combination of metformin and verteporfin synergistically inhibits cell proliferation, promotes apoptosis and suppresses cell migration/invasion by downregulating YAP, therefore reduces the side effects caused by their single use and improve the quality of life for patients with lung cancer.

Interpretation: we concluded that metformin depresses YAP promoter by interfering with the binding of the transcription factor IRF-1. Importantly, verteporfin sensitizes metformin-induced the depression of YAP and inhibition of cell growth and invasion in lung cancer cells.

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1. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide and classified into two histological subtypes: non-small-cell lung cancer (NSCLC) and small-cell lung cancer [1]. NSCLC comprises ~85% of all lung cancers [2], and despite various treatment strategies provided to patients, the prognosis remains very poor, with a 5-year survival rate of only 10% to 15% [3].

The MST-yes-associated protein (YAP) pathway was first discovered in fruit flies and is involved in regulating cancer, organ development, regeneration, and stem cell biology [4–7]. The core components of this kinase cascade include the kinases MST1/2 and LATS1/2 and the scaffold protein salvador family WW domain-containing protein 1 (SAV1) and MOB1. MST1/2 phosphorylates LATS1/2 via SAV1 in order to subsequently phosphorylate their terminal effectors, including the transcription co-factors YAP and Transcription co-activator with PDZ-binding motif (TAZ) [8]. Phosphorylated YAP/TAZ binds to the 14-3-3 protein, leading its retention in the cytoplasm and degradation by the ubiquitin-proteasome pathway. When dephosphorylated, YAP and TAZ translocate into the nucleus and interact with transcription factors, particularly TEA domain family member (TEAD), to induce target gene expression.
expression [9]. Increased expression and activation of YAP/TAZ are widespread in a broad range of cancers, including lung, liver, colon, ovary, and breast cancers [10,11]. YAP and TAZ play important roles in cancer initiation, progression, and metastasis [12]; therefore, they represent potential drug targets for cancer therapies.

Metformin was originally extracted from *Galega officinalis* and is prescribed as a first-line drug for the treatment of type 2 diabetes [13]. Metformin lowers blood glucose by decreasing hepatic gluconeogenesis, inhibiting intestinal glucose absorption, and increasing peripheral glucose uptake [14]. Growing evidence indicates the potential preventative and therapeutic anticancer effects of metformin [15]. According to an epidemiological investigation, treatment with metformin might reduce the incidence of cancer in patients with type 2 diabetes [16]. Moreover, a recent study showed that metformin use is associated with an almost 20% improvement in overall survival in patients with stage IV NSCLC [17]. Similarly, another study confirmed that metformin treatment is related to improved survival in diabetic patients after NSCLC diagnosis [18]. However, the potential mechanisms underlying the anticancer effects of metformin remain unclear, and their identification might promote the development of new therapeutic strategies.

Interferon regulatory factors (IRFs) are a group of closely related proteins collectively referred to as the IRF family. IRFs exhibit significant homology in their N-terminal region, which contains a DNA-binding domain (DBD) that includes a cluster of five tryptophan residues. This DBD forms a helix-turn-helix motif and recognizes the interferon-stimulated response element in the promoter of genes targeted by IRFs. The C-terminal region of most IRFs is less conserved and contains an IRF-response element in the promoter of genes targeted by IRFs. The proteins collectively referred to as the IRF family. IRFs exhibit significant and therapeutic anticancer effects of metformin remain unclear, and their identification might represent an effective strategy for the clinical treatment of NSCLC.

2. Materials and methods

2.1. Construction of plasmids

Myc-tagged YAP, E2F, IRF-1 and IRF-2 constructs were made using the pcDNA 3.1 vector (Invitrogen, Carlsbad, CA, USA). Sequences encoding the Myc epitope (EQKLISEEDL) were added by PCR through replacement of the first Met-encoding codon in the respective cDNA clones. The PCR primers were:

**YAP forward primer:** 5′-GGGGTACCCGCCGAGCAGAAACACTCATCTGAA GAGATCTGATGCCTCCCAGGCAGCCG-3′

**YAP reverse primer:** 5′-GCTCTAGAGCCTATAACCATGTAAGAAAGCT-3′

**E2F forward primer:** 5′-TCAGAAATCCAGGGGGGTGAG-3′

**E2F reverse primer:** 5′-ATGCCCATCACTCGGATGCCG-3′

**IRF-1 forward primer:** 5′-ATGCCCATCACTCGGATGCCG-3′

**IRF-1 reverse primer:** 5′-CTACGGTGACAGGAAATTCG-3′

**IRF-2 forward primer:** 5′-ATGCCGGTGAAGGATTGGCCG-3′

**IRF-2 reverse primer:** 5′-TTAACAGCTCTTGGACCCGG-3′.

2.2. Cell lines and culture

Human NSCLC cell lines A549, H1299, Calu6, H520 and the human lung normal control cell line HBEC-3KT (HBEC) were purchased from American Type Culture Collections (Manassas, VA). Cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS (Hyclone, USA), penicillin/streptomycin (100 μg/ml). Culture flasks were kept at 37 °C in a humid incubator with 5% CO2.

2.3. Over-expression and knockdown of genes

The over-expression plasmids (2 μg) or siRNA (1 μg) were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for over-expression or knockdown of YAP, E2F1, IRF-1 or IRF-2, followed by analysis 48–72 h later. The selected sequences for si YAP-1 were: 5′-AA-GUGGAUACUAUACAAA dTdT-3′; siYAP-2 were: 5′- AA-GACATTTCTGTCTACAGA dTdT-3′; siIRF-1-1 were: 5′-AA-GUAA UUUCCCUUCUCAUC dTdT-3′; siIRF-1-2 were: 5′-AA-GUAGAGGAGG ACAGAAAU dTdT-3′; siIRF-2 were: 5′-AA-GGACCAAAAGGGGCAGUG dTdT-3′; siE2F1 were: 5′-AA-GUGGAUACUAUACAAA dTdT-3′; and si control were 5′-AA-UUUUCCCGAAACGUCCAG dTdT-3′. These selected sequences were purchased from the company of GenePharma (Shanghai, China).

2.4. Subcellular fraction

Transfected A549 cells were harvested in PBS and resuspended for 10 min on ice in 500 μl CLB Buffer (10 mM Hepes, 10 mM NaCl, 1 mM KH2PO4, 5 mM NaHCO3, 5 mM EDTA, 1 mM CaCl2, 0.5 mM MgCl2). Thereafter, 50 μl of 2.5 M sucrose was added to restore isotonic conditions. The first round of centrifugation was performed at 6300g for 5 min at 4 °C. The pellet washed with TSE buffer (10 mM Tris, 300 mM sucrose, 1 mM EDTA, 0.1% NP40, PH 7.5) at 4000 g for 5 min
at 4 °C until the supernatant was clear. The resulting supernatant was discarded, and the pellets were nucleus. The resulting supernatant from the first round of differential centrifugation was sedimented for 30 min at 14000 rpm. The resulting pellets were membranes and the supernatant were cytoplasm.

2.5. Western blot analysis

Human lung cancer cells with treatment of metformin were transfected with the relevant plasmids and cultured for 36 h. For western blot analysis, cells were lysed in NP-40 buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 1 mM PMSF, and 0.5% NP-40) at 25 °C for 40 mins. The lysates were added to 5× loading dye and then separated by electrophoresis. The primary antibodies used in this study were 1:1000 rabbit anti-Myc (Santa Cruz, Dallas, TX, USA), 1:1000 anti-YAP, anti-pYAP, anti-CTGF and anti-Cyr61 (Cell Signaling Technology, Danvers, MA, USA), 1:1000 anti-E2F1, anti-IRF-2, anti-cytochrome c, anti-Bcl-2, anti-Bcl-XL, anti-PARP anti-Vimentin, anti-E-cadherin and anti-cleaved caspase-3 (Abcam, Cambridge, UK). The images were obtained through Image Lab™ Software (ChemiDoc™ XRS+). E-cadherin forward primer: 5′-AAGCCACATCATGCAAGGTCG-3′. E-cadherin reverse primer: 5′-CTGGTCACCCTGGCCCGCAG-3′. IRF-1 forward primer: 5′-AAAGTGAATCCAGCCGAG-3′. IRF-1 reverse primer: 5′-CAGAGTGGACTGCTGATGCT-3′. IRF-2 forward primer: 5′-TGTGATCCGCTGCTGAA-TG-3′. IRF-2 reverse primer: 5′-AAGCATGCGTGGGACAT-CA-3′. E2F1 forward primer: 5′-ATCCAGAAGTCCTTA CTACA-3′. E2F1 reverse primer: 5′-ACC GCAGGGTCGAGCATCCC-3′. GAPDH forward primer: 5′-CTTCTCTGTTGTGCACGATGC-3′. GAPDH reverse primer: 5′-CCTAA TACCAACAAATCCGT-T-3′.

2.9. Colony forming assay

For colony formation assay, A549 cells with indicated treatment were added to growth medium in RPMI-1640 medium supplemented with 10% FBS (HyClone, USA), penicillin/streptomycin (100 mg/ml). Culture flasks were kept at 37 °C in a humid incubator with 5% CO2. Cells were fed with 1 ml of medium every three days. The colonies were stained with 0.01% crystal violet and counted in 2–3 weeks. The results were observed under an optical microscope (IX53; Olympus) and assessed using the Image J software.

2.10. SA-β-gal staining

SA-β-gal was detected using the Senescence (β-Galactosidase Staining kit (C0602; Beyotime) following the manufacturer’s instructions: In brief, the cells were washed twice with PBS and then fixed with PBS containing 2% formaldehyde and 0.2% glutaraldehyde for 10 mins. The cells were then incubated at 37 °C for 12 h with staining solution. After being washed twice with PBS, the SA-β-gal-positive cells were observed under an optical microscope (IX53; Olympus) and assessed using the Image J software.

2.11. MTT and CCK8 assays

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and CCK8 assays in 96-well plates in a manner similar to that previously described for the CCK8 assays. Cells were transfected with indicated plasmids 48 h and with indicated treatment, followed by incubation with MTT and CCK8 for 4 h. Next 100 μl dimethyl sulfoxide was added to dissolve the formazan crystals for the MTT assay. Absorbance was read at 570 nm for MTT and 450 nm for CCK8 using a spectrophotometer (Tecan, Männedorf, Switzerland). Cell viability was calculated as relative absorbance compared to a DMSO-only control.

2.12. luciferase reporter assay

To construct the core region of YAP promoter, the region of YAP was amplified by PCR from the human cDNA of A549 cells and was inserted into the upstream of the pGL3-Basic vector (Promega, Madison, WI, USA) via KpnI and XhoI sites to generate YAP luc. Thereafter, we use the Firefly Luciferase Reporter Gene Assay Kit (Beyotime, RG005) to detect the promoter activities. The PCR primers were: YAP forward primer: 5′-GATACACCGGGCTAGGAA-3′. YAP reverse primer: 5′-GATACACCGGGCTAGGAA-3′.

2.6. Immunofluorescent staining

To examine the protein expressions of YAP, CTGF, Ki67, Vimentin, E-cadherin and Annexin V, lung cancer cells were seeded onto coverslips in a 24-well plate and then transfected with indicated plasmids and treated with metformin for 36 h. Cells were then fixed using 4% formaldehyde for 30 min at 25 °C and treated with 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 30 mins. The coverslips were incubated with rabbit anti-YAP, CTGF, Ki67, Vimentin, E-cadherin and Annexin V monoclonal antibody (Cell Signaling Technology) at 1:200 dilution in 3% BSA. The coverslips were then incubated with an Alexa Fluor 488 (green) and Alexa Fluor 594 (red) tagged anti-rabbit or anti-mouse monoclonal secondary antibody at 1:1000 dilution in 3% BSA. DAPI (3 μg/ml) was added for nuclear counterstaining. Images were obtained with a Zeiss Axio Imager Z1 Fluorescent Microscope (Zeiss, Oberkochen, Germany).

2.7. Cell flow cytometry assay

A549 cells with indicated treatment were harvested and fixed with 70% ethanol. These cells were then stained using propidium iodide (PI) and the cell cycle stage assessed by flow cytometry. To evaluate apoptosis, cells were cultured at 80% confluence and trypsinized and assessed using the Image J software.

2.8. RNA isolation, RT-PCR and RT-qPCR assay

We used Trizol reagent (TransGen Biotech, Beijing, China) to isolate total RNA from the samples and cells. RNA was reverse transcribed into first-strand cDNA using a TransScript All-in-One First-Strand cDNA Synthesis Kit (TransGen Biotech.). CDNAs were used in RT-PCR and RT-qPCR assay with the human GAPDH gene as an internal control. The final RT-qPCR reaction mixture contained 10 μl Bestar® SYBR Green qPCR Master Mix. Amplification was performed as follows: a denaturation step at 94 °C for 5 mins, followed by 40 cycles of amplification at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. The reaction was stopped at 25 °C for 5 mins. The relative expression levels were detected and analyzed by ABI Prism 7900HT/FAST (Applied Biosystems, USA) based on the formula of 2ΔΔCT. We got the images of RT-PCR by Image Lab™ Software (ChemiDoc™ XRS+). E-cadherin forward primer: 5′-AAGCCACATCATGCAAGGTCG-3′. E-cadherin reverse primer: 5′-CTGGTCACCCTGGCCCGCAG-3′. IRF-1 forward primer: 5′-AAAGTGAATCCAGCCGAG-3′. IRF-1 reverse primer: 5′-CAGAGTGGACTGCTGATGCT-3′. IRF-2 forward primer: 5′-TGTGATCCGCTGCTGAA-TG-3′. IRF-2 reverse primer: 5′-AAGCATGCGTGGGACAT-CA-3′. E2F1 forward primer: 5′-ATCCAGAAGTCCTTA CTACA-3′. E2F1 reverse primer: 5′-ACC GCAGGGTCGAGCATCCC-3′. GAPDH forward primer: 5′-CTTCTCTGTTGTGCACGATGC-3′. GAPDH reverse primer: 5′-CCTAA TACCAACAAATCCGT-T-3′.
2.13. CHIP assay

ChIP experiments were performed according to the laboratory manual. Immunoprecipitation was performed for 6 h or overnight at 4 °C with specific antibodies. After immunoprecipitation, 45 μl protein A-Sepharose and 2 μg of salmon sperm DNA were added and the incubation was continued for another 1 h. Precipitates were washed sequentially for 10 min each in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), and buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Precipitates were then washed three times with TE buffer and extracted three times with 1% SDS, 0.1 M NaHCO3. Eluates were pooled and heated at 65 °C for at least 6 h to reverse the formaldehyde cross-linking. DNA fragments were purified with a QIAquick Spin Kit (Qiagen, CA). For PCR, 1 μl from a 50 μl extraction and 21–25 cycles of amplification were used. We got the images of PCR by Image Lab™ Software (Chemidoc™ XRS+, BIO-RAD), and these images were TIF with reversal color format. The sequences of the primers used are provided as follows:

YAP forward primer: 5′-CTCTGTITTTCCAGTTG-3′.
YAP reverse primer: 5′-AGGGTGAAAGAATCCAT-3′.

2.14. Wound healing assays

To assess the cellular migration, 10⁴ cells were seeded onto 6-well plates with transfection of the relevant plasmids. These cells were then incubated in 5% CO₂ at 37 °C for 48 h. A wound was scraped into the cells using a plastic 200 μl tip and then washed with PBS. The cells were then incubated in DMEM containing 10% FBS. Images were captured at the time points of 0 and 36 h after wounding. The relative distance of the scratches was observed under an optical microscope (IX53, Olympus, Tokyo, Japan) and assessed using the Image J software.

2.15. Transwell invasion assays

Transwell invasion assays were performed using a 24-well chamber (Costar 3422; Corning Inc., Corning, NY, USA). The lower and upper chambers were partitioned by a polycarbonate membrane (8-μm pore size). Lung cancer cells (1 × 10³) were seeded into DMEM without FBS in the upper chamber. DMEM containing 10% FBS was added to the lower chamber. The cells were allowed to migrate for 36 h at 37 °C in a humidified atmosphere containing 5% CO₂. Cells remaining on the upper side of the membrane were removed using PBS-soaked cotton swabs. The membrane was then fixed in 4% paraformaldehyde for 20 min at 37 °C and then stained with crystal violet. Cells on the lower side of the membrane were counted under an Olympus light microscope (Olympus, Tokyo, Japan) and assessed using the Image J software.

2.16. Measurement of superoxide production

The O₂⁻ dependent oxidation in A549 cells treated with 1 μM MitoCP or co-treated with 1 μM Mito-CP and 2 μg YAP in the presence of 7800 units/ml catalase was detected spectrophotometrically at 486–575 nm with a dual wavelength spectrophotometer (Tecan, Männedorf, Switzerland). The experiment process and parameters were described in MitoSOX™ Red mitochondrial superoxide indicator (M36008, Invitrogen, US).

2.17. UV-visible absorption spectral measurements

The UV-visible absorption spectra were recorded to investigate the extracellular interaction between DNA and metformin using UV-visible spectrophotometer and 1-cm quartz cuvette (T60, PG Instruments Ltd., Leicestershire, UK). The UV-visible absorption spectra of DNA in the presence of metformin was measured in the range of 240 nm–300 nm at room temperature described in the Khajeh et al. [21].

2.18. Fluorescence polarization–based peptide displacement assay

This assay is designed based on the concept that binding of a fluorescein-labeled peptide to a larger molecule, such as a nucleic acid, results in a higher fluorescence polarization (FP) signal that will decrease upon displacement of the peptide by small molecules. The Kdisp values were calculated using the four parameters logistic equation using GraphPad. The equation is as follows:

\[ P = P_{\text{min}} + \left( \frac{P_{\text{max}} - P_{\text{min}}}{1 + (X/K_{\text{disp}})^H} \right) \]

where \( P \) stands for polarization; \( P_{\text{min}} \), minimum polarization; \( P_{\text{max}} \), maximum polarization; and \( H \), Hill slope. The N-terminal IRF-1 labeled with fluorescein isothiocyanate (FITC) (N-IRF-1) was purified in A549 cells. The metformin/IRF-1 response element of the YAP was synthetized (Sangon, Shanghai, China) and the compound binding assays were performed in a 10 μl volume at a constant concentration of 3.0 × 10⁻⁵ mol/L. The assay buffer was 20 mM Tris, pH 8.8, 50 mM NaCl, and 0.01% Triton X-100. FP assays were performed in 384-well plates using a Synergy 4 microplate reader (Tecan, Männedorf, Switzerland). An excitation wavelength of 485 nm and an emission wavelength of 528 nm were used. The experiment process and parameters were described in Senisterra G. et al. [22].

2.19. Human lung cancer specimen collection

All the human lung cancer and normal lung specimens were collected in Affiliated Hospital of Binzhou Medical College with written consents of patients and the approval from the Institute Research Ethics Committee.

2.20. Analysis of publicly available datasets

To analyze correlation between Yap expression level and prognostic outcome of patients, Kaplan-Meier survival curves of NSCLC patients with low and high expression of YAP were generated using Kaplan-Meier Plotter (www.kmplot.com/analysis) [23].

2.21. In vivo experiments

To assess the in vivo effects of metformin, 3 to 5-week old female BALB/c athymic (NU/NU) nude mice were housed in a level 2 biosafety laboratory and raised according to the institutional animal guidelines of Binzhou Medical University. All animal experiments were carried out with the prior approval of the Binzhou Medical University Committee on Animal Care. For the experiments, mice were injected with 5 × 10⁶ lung cancer cells and randomly divided into two groups (five mice per group) after the diameter of the xenografted tumors had reached approximately 5 mm. Xenografted mice were then administrated with PBS or metformin (orally, 250 mg/kg per day), and tumor volume and body weight were measured every second day. For the in vivo study of the synergistic effects of metformin and YAP inhibitor VP, BALB/c mice (n = 10) were also provided a daily oral dose of –250 mg/kg metformin or injected with Verteporfin (5 mg/Kg) individually or combined use these two drugs. Tumor volume was estimated as 0.5 × a² × b (where a and b represent a tumor’s short and long diameter, respectively). Mice were euthanized after six weeks of treatment and the tumors were measured at a final time. Tumors were then collected from xenograft mice and analyzed by immunohistochemistry and Western blot.
2.22. Feeding experiment

The female BALB/c athymic (NU/NU) nude mice were kept under the standard laboratory conditions of the animal facilities of Binzhou Medical University Hospital in a temperature (22 ± 2 °C) and humidity (45% to 50%) controlled room. In order to investigate the synergistic effects of metformin and YAP inhibitor VP, the mice (n = 10) were divided into four groups and provided an individual daily oral dose of ~250 mg/kg metformin or injected with Verteporfin (5 mg/Kg) or combined use these two drugs three times a week. Their food intake was detected (measured by weighing the consumption of food for mice) at 6 h after treatment mentioned above.

2.23. Immunohistochemical analysis

Tumor tissues were fixed in 4% paraformaldehyde overnight and then embedded in paraffin wax. Four-micrometer thick sections were stained using hematoxylin and eosin (H&E) for histological analysis according to the laboratory manual.

2.24. Combined index (CI) of drug assay

The combined index of drug interaction (CI) was used to analyze the synergistically inhibitory effect of drug combinations. CI is calculated as follows: $CI = AB/(AXB)$. According to the absorbance of each group, AB is the ratio of the combination groups to the control group; A or B is the ratio of the single agent groups to the control group. Thus, a CI value less than, equal to or >1 indicates that the drugs are synergistic, additive or antagonistic, respectively. CI <0.7 indicate that the drugs are significantly synergistic.

2.25. Small molecule microarray

ChemBank molecular library (http://chembank.broad.harvard.edu) is a public, web-based informatics environment developed through a collaboration between the Chemical Biology Program and Platform at the Broad Institute of Harvard and MIT. The selected sequences (−56−7), which includes the IRF-1/metformin-response element within YAP promoter, were synthetized from the company of GenePharma (Shanghai, China). Typically, each small molecule of ChemBank molecular library in the detection board was mixed with the DNA element (10 μM), which is amine-modified at the 5’ end and allows to produce detection signal. The mixture was concentrated in vacuo. The vials containing the mixtures were irradiated at 365 nm for 1 h with a Super-light model then re-dissolved in MeOH and analyzed with a Perkin–Elmer SCIEX API 2000 pneumatically assisted electrospray triple-quadrupole mass spectrometer equipped with a Hewlett–Packard Series 1100 HPLC system column: 200 mm × 2 mm PEGASIL ODS column (Senshu Scientific Co., Ltd. Tokyo, Japan). The detailed methods of screening for molecular library and data were analyzed in the small molecule microarray according protocols described previously in Seiler and Dominik [24,25].

2.26. Mass spectrometry conditions

Single-stranded oligonucleotides of YAP promoter (−56−7) were purchased from Sangon (Beijing, China). Oligonucleotides were dissolved in water and synthetized for duplex DNA then desalted for three times. A haloform reaction was used to synthesize the building blocks that contained two to four heterocycle rings, these building blocks were then joined by means of the DCC/HOBt coupling reaction to produce the polyamides. Polyamides were dissolved with methanol/100 mm ammonium acetate (20:80, v/v) to a final volume of 40 ml. These mixtures were incubated with the ChemBank molecular library and then adsorbed and eluted with methanol. Mass spectrometry experiments were performed on a high-resolution quadrupole orbitrap tandem mass spectrometer with a heated electrospray (HESI) probe (Q-Exactive, Thermo Fisher Scientific, Waltham, MA). The ESI source parameters were as follows (positive/negative ion mode): sheath gas 40/45, auxiliary gas 11/10, auxiliary gas heater temperature 220 °C, spray voltage 3.5/−3.2 kV, Capillary temperature 350 °C. Alternating full MS scan and MS/MS scans were 1 implemented in both PRM and AIF assays. The detailed experiment process and parameters were described in Gao, et al. [26].

2.27. Ethics statement

The experimental protocol was approved by the Research Ethics Committee of Binzhou Medical University, China (No. 2017-014-01 for human lung cancer specimen and No. 2017-004-09 for mouse experiments in vivo) and the written informed consent was obtained from all subjects. Informed consent was obtained from all individual participants included in the study.

2.28. Statistical analysis

Each experiment was repeated at least three times. The statistical analyses of the experiment data were performed by using a two-tailed Student’s paired t-test and one-way ANOVA. Statistical significance was assessed at least three independent experiments and the P-value <0.05 was considered statistically significant and highlighted an asterisk in the figures, while P-values <0.01 were highlighted using two asterisks and P-values <0.001 highlighted using three asterisks in the figures.

3. Results

3.1. Aberrant activation of YAP in lung tumors is associated with adverse prognosis

A total of 60 samples were obtained from patients who underwent lung-resection surgery at the Affiliated Hospital of Binzhou Medical College (Binzhou, China) between January 2015 and January 2017. Each sample was examined, with the clinicopathologic findings summarized in Table 1. We first examined YAP expression in lung tissues from patients with NSCLC by reverse transcription polymerase chain reaction (RT-PCR), western blot, RT-qPCR, and immunohistochemistry. Fig. 1a through d shows that YAP mRNA and protein levels were elevated in NSCLC tissues as compared with levels in normal adjacent lung tissues analyzed by RT-PCR (Fig. 1a), western blot (Fig. 1b), RT-qPCR (Fig. 1c), and immunohistochemical staining of frozen sections (Fig. 1d). Additionally, we confirmed that YAP mRNA and protein levels in a panel of NSCLC cell lines, including A549, H1299, Calu6, and H520 cells, was significantly higher than that in normal HBEC cells (Fig. 1e–h). The expression of two YAP-specific downstream target genes, connective tissue growth factor (CTGF) and cysteine-rich angiogenic inducer 61 (Cyr61), was also increased in the NSCLC cell lines (Fig. 1e–g), indicating that YAP was hyperactivated, whereas the phosphorylation level of YAP was lower in NSCLC cells as compared with controls cell HBEC (Fig. 1g and j). Consistently, YAP protein was observed primarily in the nucleus in NSCLC cells as compared with cytosol in HBEC cells according to immunofluorescence staining (Fig. 1h and j) and western blot (Fig. 1i) analysis. Publicly available datasets (http://www.kmplot.com/analysis/index.php?p=service&cancer=lung; 2015 version) [23,27,28] were screened to analyze prognostic correlations between YAP expression and the survival of patients with lung cancer. Kaplan–Meier analysis indicated that elevated YAP expression inversely correlated with overall survival (OS; p = 1.1e − 0.9 according to the log-rank test for significance; n = 1926) (Fig. 1k). These results indicated that YAP expression and activity correlate with NSCLC.
using siYAP-2 was better (Supplementary Fig. 1a). The results showed that the proliferative ability of the cells positively correlated with YAP expression, we study and compare the effects of different protein levels of YAP in lung cancer cell properties, we established a control (co-transfection of pcDNA vector), and Myc-YAP (co-transfection of pcDNA vector encoding Myc-YAP) and an siRNA control) groups for each experiment. To investigate the role of YAP in cell growth, we performed assays using a Cell Counting Kit 8 (CCK8) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), evaluated the rate of Ki-67-positive staining, and pGL3-185/+/95 region represented the core region of YAP promoter. Regions of YAP promoter. Regions of YAP promoter (−1050/+105, −400/+90, and −90/+95) were cloned in the pGL3 plasmid to generate pGL3-1300, pGL3-310, and pGL3-185 plasmids, which were used in luciferase reporter assays in A549 cells to determine their activity with the treatment of 10 mM metformin for 60 h. We found pGL3-185 exhibited minimal luciferase activity among the three constructs (Fig. 3a), indicating that metformin treatment decreased pGL3-185-related luciferase activity in A549 cells (Fig. 2i). We then examined the effect of YAP on cell migration and invasion via scratch and transwell assays, respectively. We found that YAP expression was positively associated with the migratory and invasive abilities of A549 cells (Fig. 2j and k). These data suggested that YAP is critical for the regulation of cancer-cell proliferation, apoptosis, senescence, migration, and invasion. To assess the role of YAP in EMT, we variations in E-cadherin and vimentin levels according to the results that metformin inactivates YAP in A549 and H1299 cells. We further investigated the effect of metformin on YAP promoter activity using a luciferase reporter assay, finding that metformin significantly decreased transcriptional activation of YAP in A549 and H1299 cells (Fig. 3i). Moreover, immunofluorescence staining and western blot analysis demonstrated that metformin decreased YAP levels in the nucleus and increased phosphorylated YAP levels in the cytoplasm of A549 and H1299 cells (Fig. 3j and k), with YAP consistently found in cytoplasmic extracts from A549 cells treated with metformin and in nuclear extracts from untreated A549 cells (Fig. 3k). These data indicated that metformin suppressed YAP activity in lung cancer cells by downregulating YAP mRNA expression and augmenting YAP phosphorylation, thereby inhibiting YAP nuclear translocation and target gene expression.

3.3. Metformin inhibits YAP activity in lung cancer cells

Because the effects of YAP knockdown were similar to cellular responses to metformin, we speculated that the effect of metformin is at least partially exerted through its action on YAP signaling. To determine whether YAP plays a role in responses to metformin, we measured the expression of YAP and its target genes following treatment with increasing concentrations of metformin (range: 0–20 mM). In A549 or H1299 cells treated with metformin, YAP mRNA and protein levels, as well as levels of the YAP target genes CTGF and Cyr61, significantly decreased along with increases in YAP phosphorylation in a dose-dependent manner according to RT-qPCR, western blot (Fig. 3a–d), and RT-PCR (Supplementary Fig. 2a–d) analyses. Similarly, as shown in the Fig. 3e–h and Supplementary Fig. 2e–h, metformin treatment downregulated YAP, CTGF, and Cyr61 expression and enhanced YAP phosphorylation over time, confirming that metformin inactivates YAP in A549 and H1299 cells. We further investigated the effect of metformin on YAP promoter activity using a luciferase reporter assay, finding that metformin significantly decreased transcriptional activation of YAP in A549 and H1299 cells (Fig. 3i). Moreover, immunofluorescence staining and western blot analysis demonstrated that metformin decreased YAP levels in the nucleus and increased phosphorylated YAP levels in the cytoplasm of A549 and H1299 cells (Fig. 3j and k), with YAP consistently found in cytoplasmic extracts from A549 cells treated with metformin and in nuclear extracts from untreated A549 cells (Fig. 3k). These data indicated that metformin suppressed YAP activity in lung cancer cells by downregulating YAP mRNA expression and augmenting YAP phosphorylation, thereby inhibiting YAP nuclear translocation and target gene expression.

3.4. Metformin attenuates YAP promoter activity by interfering with IRF-1 binding

To investigate the effect of metformin on YAP expression, we first isolated the core region of YAP promoter. Regions of YAP promoter (−1050/+105, −400/+90, and −90/+95) were cloned in the pGL3 plasmid to generate pGL3-1300, pGL3-310, and pGL3-185 plasmids, which were used in luciferase reporter assays in A549 cells to determine their activity with the treatment of 10 mM metformin for 60 h. We found pGL3-185 exhibited minimal luciferase activity among the three plasmids (Fig. 4a), indicating that the −90/+95 region represented the core region of YAP promoter repressed by metformin. We then observed that metformin treatment decreased pGL3-185-related luciferase activity in A549 cells in a dose-dependent manner (Fig. 4b), suggesting that this region of YAP promoter might be responsible for metformin-mediated attenuation YAP promoter activity. Subsequent

### Table 1
Patient’s demographics and tumor characteristics and association of YAP level with clinicopathological features in lung cancer population.

| Characteristics | No. of patients, N = 60 (%) | P value |
|-----------------|----------------------------|---------|
| Age (years)     |                            | 0.152   |
| Average [range] | 55 [30–81]                 |         |
| <55             | 20 (33.3)                  |         |
| ≥55             | 40 (66.7)                  |         |
| Gender          |                            | 0.0581  |
| Male            | 35 (58.3)                  |         |
| Female          | 25 (41.7)                  |         |
| Tumor Characteristics |              |         |
| Tumor size (cm) |                            |         |
| <4              | 12 (20.0)                  | 0.006** |
| ≥4              | 48 (80.0)                  |         |
| Differentiation |                            |         |
| Poor            | 40 (66.7)                  | 0.056   |
| Well-moderate   | 20 (33.3)                  |         |
| Lymph node metastasis |                |         |
| N−              | 26 (43.3)                  | 0.004** |
| N+              | 34 (56.7)                  |         |
| Distal metastasis |                           |         |
| M−              | 28 (46.7)                  | 0.008** |
| M+              | 32 (53.3)                  |         |
| Level of YAP    |                            |         |
| Protein level   | N = 35 (Fig. 1B)           |         |
| High            | 29 (83.0)                  | 0.001** |
| Median          | 3 (8.5)                    | 0.031†  |
| Low             | 3 (8.5)                    | 0.142   |
| mRNA level      | N = 35 (Fig. 1A)           |         |
| High            | 30 (85.7)                  | 0.001** |
| Median          | 3 (8.5)                    | 0.033†  |
| Low             | 2 (5.8)                    | 0.157   |

Differences between experimental groups were assessed by Student’s t-test or one-way analysis of variance. Data represent mean ± SD.

* p < 0.05
** p < 0.01.
we analyzed the potentially bind this region of the transcription factors, including c-Ets-1, c-Ets-2, Elk-1, and IRF-1, could v3/promo/promo.cgi?dirDB=TF_8.3). Interestingly, we found that four the PROMO platform [29,30] (http://alggen.lsi.upc.es/cgi-bin/promo_ bition of metformin-response element of the GGA). These data con dication a direct interaction between metformin and DNAp (TTTCCT in along with a slight red shift in the maximum emission wavelength, in- tration, we observed a gradual quenching of visible absorption spectral measurements. The absorbance value at 260 nm (A260 nm) compared with the concentration ratio of metfor- min to DNAp was 0.43 at the in- tervention point of the measurement curve (Fig. 4d). As shown in Fig. 4e, upon increasing the DNAp concentration, we observed a gradual quenching of fluorescence intensity along with a slight red shift in the maximum emission wavelength, indicating a direct interaction between metformin and DNAp (TTTCTT GGA). These data confirmed that metformin directly bound the metformin-response element of the YAP promoter (−36 to −27).

To identify the mechanism associated with metformin-specific inhibition of YAP promoter activity, we analyzed the −90/+95 region using the PROMO platform [29,30] (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promo.cgi?dirDB=TF_8.3). Interestingly, we found that four transcription factors, including c-Ets-1, c-Ets-2, Elk-1, and IRF-1, could potentially bind this region of the YAP promoter (Fig. 4f). Moreover, we analyzed the −400/~90 region of the YAP promoter, the luciferase activity of which was similar to that of the luciferase control (Fig. 4a).

Recent reports indicate that E2F within this region of the YAP promoter is modulated by metformin and cooperates with YAP/TEAD2 to promote a cell cycle progression and DNA replication [31,32]. Therefore, we explored whether E2F could be modulated by metformin to regulate YAP expression. Our results showed that the activity of the YAP promoter and YAP expression were not regulated by E2F in A549 cells (Supplementary Fig. 4a–d), indicating that metformin-mediated attenuation of YAP promoter activity is independent of E2F. Importantly, we found that three of the transcription factors (c-Ets-1, c-Ets-2, c-Ets-1, and Elk-1) also continued to bind the −400/~90 region of the YAP promoter (Fig. 4f), suggesting that they were not involved in YAP promoter activation (i.e., region −90/+95). A previous study reported that IRF-1 upregulation contributes to malignant transformation by regulating genes participating in multiple aspects of human cancer initiation, progression, and metastasis. To identify a relationship between IRF-1, metformin, and the YAP promoter, we constructed luciferase reporter plasmids containing both the wild-type YAP promoter (~90+/+95; YAPLuc-Wt) or a YAP promoter harboring a mutated version (~36 to −27) of the metformin-response element (YAPLuc-Mut) (Fig. 4c). Additionally, we established IRF-1 knockdown (siIRF-1-1 and siIRF-1-2) (Supplementary Fig. 3b) or overexpressing cell lines by transfection of pcDNA-IRF-1 in A549 cells (Fig. 4g). We found that knockdown of IRF-1 using siIRF-1-2 was better (Supplementary Fig. 3b–f), therefore siIRF-1-2 was used to carry out the experiment for knockdown of IRF-1. Co-transfection of A549
cells with YAPLuc-Wt and the IRF-1-overexpressing plasmid increased luciferase activity relative to that observed with control plasmids. Similarly, co-transfection with YAPLuc-Wt and siIRF-1 decreased the luciferase activity in A549 cells relative to co-transfection with the control plasmid and siRNA. By contrast, luciferase activity did not change in A549 cells co-transfected with YAPLuc-Mut and with either status of IRF-1 (Fig. 4h). These results suggested that IRF-1 directly targets the YAP promoter at the metformin-response element. Moreover, we observed that IRF-1 bound the YAP promoter and increased YAP transcription and that of its target genes. Evaluation of IRF-2, an IRF-1 homolog, was performed according to reports showing differences in amino acid composition and biological function between IRF-1 and IRF-2 [33,34]. Importantly, we analyzed the core region of YAP promoter using the PROMO platform and we found that IRF-2 did not bind this region of the YAP promoter [29,30] and confirmed that YAP expression was not regulated by IRF-2 (Supplementary Fig. 4e–h). We then assessed whether competitive

Fig. 2. YAP is involved in lung cancer-cell proliferation, apoptosis, migration, invasion, and EMT. A549 cells were knockdown of YAP by using siRNA or overexpressed by using Myc-YAP. a, b the mRNA and protein levels of YAP were analyzed by RT-PCR, western blot (a) and RT-qPCR (b) assay. c the cellular proliferation was analyzed by CCK8 and MTT assay. d the protein of Ki67 was analyzed by immunofluorescent staining. e the Edu staining cells were analyzed by immunofluorescent staining. f the protein of cleaved Caspase3 was analyzed by immuno blotting assay. g the protein of Annexin V was analyzed by immunofluorescent staining. h Colony formation density was analyzed by colony formation assay. i Cell senescence was analyzed by SA-β-gal staining. j Cellular migration ability was analyzed by cell scratch assay. k Cellular invasion ability was analyzed by transwell assay. l The expression of E-cadherin and Vimentin were analyzed by RT-PCR, western blot and RT-qPCR assay. m Statistical analysis of the protein expression levels of YAP with E-cadherin/Vimentin was analyzed based on western blot assay in A549 cells. Results were presented as mean ± SD, and the error bars represent the SD of three independent experiments. (*p < 0.05, **p < 0.01, ANOVA with Bonferroni correction).
binding occurred between metformin and IRF-1 in this region, potentially resulting in metformin-mediated inhibition of YAP expression and that of its target genes. ChIP assays revealed that IRF-1 bound the YAP promoter in the absence of metformin but was undetectable upon treatment of the cells with metformin (Fig. 4m). To identify whether metformin disrupts IRF-1 interaction with the YAP promoter, we firstly detected the effect of metformin on the IRF-1. Our result showed that the expression of IRF-1 was not regulated by metformin by the RT-qPCR (Supplementary Fig. 4i) and Western blot assay (Supplementary Fig. 4j), which indicated that metformin neither binds to nor regulates the expression of IRF-1 in A549 cells. We then used fluorescein isothiocyanate-labeled IRF-1 (1–120) peptides that included the DNA-binding domain to perform peptide-displacement assays (Supplementary Fig. 3k) [22,35]. After verifying IRF-1 peptide (1–120) binding to the YAP promoter (Supplementary Fig. 3l), we found that metformin treatment displaced the labeled peptide with a $K_{\text{disp}}$ value of 10 ± 2.3 mM (Supplementary Fig. 3m), indicating that metformin interfered with IRF-1 binding to the YAP promoter. Moreover, we found that metformin treatment abrogated the increased YAP promoter activity induced by IRF-1 in A549 cells in a dose-dependent manner (Fig. 4n). Furthermore, we used the small-molecule microarray, which are collections of small drug molecules arrayed on modified glass slides and provide a fast and inexpensive method for identifying small drug molecules that bind to specific molecules such as protein, DNA, RNA, etc. [24,25], to identify whether metformin directly binds to the IRF-1 response element within the YAP promoter. Our result showed that the wild-type YAP promoter (−90/+95) directly binds to the small molecule of metformin (Supplementary Fig. 3n). In addition, mass spectrometry analysis indicated that the eluent achieved from IRF-1 response element contains the small molecule of metformin (Supplementary Fig. 3o). These findings indicated that metformin interfered with IRF-1 binding to the YAP promoter and inhibited YAP expression.
3.5. Metformin inhibits cell proliferation, arrests cell cycle progression, and promotes cell senescence and apoptosis in lung cancer cells

We then investigated the effect of metformin on several biological processes in A549 cells. CCK8 and MTT assays demonstrated that metformin inhibited cell growth in a dose- and time-dependent manner (Fig. 5a). A decrease in the number of Ki-67-positive cells was observed in cells treated with metformin (Fig. 5b), and anchorage-independent colony formation assays showed that the number of A549 colonies decreased upon treatment with metformin in a dose-dependent manner (Fig. 5c). We further examined the cell cycle distribution of A549 cells treated with metformin by flow cytometry, finding that metformin treatment reduced the proportion of cells in the S phase and enhanced that of cells in the G2 phase (Fig. 5d). Moreover, A549 cells treated with metformin showed increased cell senescence, as shown by β-galactosidase staining (Fig. 5e), cleaved caspase-3 (Fig. 5f) and Annexin V (Fig. 5g) levels, and apoptosis levels according to flow cytometry (Fig. 5h). Furthermore, metformin inhibited cell invasion and migration (Fig. 5i and Supplementary Fig. 5a). We then determined whether metformin regulated the expression of E-cadherin and vimentin, finding that
metformin treatment upregulated E-cadherin expression, but down-regulated vimentin in a dose-dependent manner (Fig. 5j and k; and Supplementary Fig. 5b). These results indicated that metformin treatment inhibited cell proliferation, arrested cell cycle progression, and promoted cell senescence and apoptosis in lung cancer cells.

3.6. Metformin interferes with YAP-mediated NSCLC initiation, progression, and metastasis

Our results showed that YAP regulates cell proliferation, apoptosis, and cell senescence (Fig. 2), and that metformin treatment attenuated YAP activity in lung cancer cells (Figs. 3 and 4) and decreased cell proliferation, invasion, migration, and EMT while increasing cell senescence and apoptosis (Fig. 5). Therefore, we hypothesized that metformin treatment would interfere with YAP-mediated NSCLC initiation, progression, and metastasis in lung cancer and investigated whether YAP downregulation at the transcription level could account for the effect of metformin on the aforementioned processes. Previous reports showed that AMP-activated protein kinase (AMPK) phosphorylation is significantly increased in response to metformin treatment, and that activation of AMPK leads to YAP cytoplasmic retention and S127

Fig. 5. Metformin inhibits cell proliferation, arrests cell cycle progression, and promotes cell senescence and apoptosis in lung cancer cells. The A549 cells were treated with indicated concentration of metformin for 60 h. a The cellular proliferation was analyzed by CCK8 and MTT assay. (*p < 0.05, **p < 0.01, ***p < 0.001, ANOVA with Bonferroni correction). b The protein of Ki67 was analyzed by immunofluorescent staining. (***p < 0.001 vs control group correspond to two-tailed Student’s tests). c Colony formation density was analyzed by colony formation assay. (***p < 0.001 vs control group correspond to two-tailed Student’s tests). d Cell cycle profile was analyzed by cell flow cytometry. (***p < 0.001 vs control group correspond to two-tailed Student’s tests). e Cell senescence was analyzed by SA-β-gal staining. (***p < 0.001 vs control group correspond to two-tailed Student’s tests). f The protein of cleaved Caspase3 was analyzed by immunoblotting assay. g The protein of Annexin V was analyzed by immunofluorescent staining. h The apoptosis was analyzed by cell flow cytometry. i Cellular invasion ability was analyzed by cell transwell assay. (**p < 0.01 vs control group correspond to two-tailed Student’s tests). j, k The expression of E-cadherin and Vimentin were analyzed by RT-PCR, western blot (j) and immunofluorescent staining (k). Results were presented as mean ± SD, and the error bars represent the SD of three independent experiments.
phosphorylation [36–39]. Therefore, to evaluate which YAP-inhibitory function dominated in lung cancer cells, we separately transfected A549 cells with plasmids overexpressing YAP or YAPS127A (the continuously activated form of YAP), followed by metformin administration. Our results showed no clear changes in cell growth (Fig. 6a), Ki-67 staining (Supplementary Fig. 6a), cleaved caspase-3 levels (Supplementary Fig. 6b), or cell migration (Supplementary Fig. 6c) and invasion (Supplementary Fig. 6d) between groups, suggesting that the inhibitory effect of metformin on YAP at the transcription level is more important than the phosphorylation status of YAP in A549 cells. Furthermore, we treated YAP-knockdown or -overexpressing cells with metformin and observed that lung cancer-cell proliferation significantly increased after YAP overexpression, but that this effect was suppressed upon metformin treatment (Fig. 6a and b; and Supplementary Fig. 6e).

Additionally, lung cancer-cell proliferation significantly decreased after YAP silencing, with this effect augmented by metformin treatment (Fig. 6b and Supplementary Fig. 6e). Similar results were obtained when we investigated the expression of Ki67 (Fig. 6c), Edu interpolation (Supplementary Fig. 6f), colony formation (Fig. 6d), cell senescence (Fig. 6e), cell migration (Fig. 6f), and cell invasion (Fig. 6g) upon YAP overexpression or silencing and cotreatment of them with metformin. Moreover, assessment of E-cadherin and vimentin regulation by YAP following metformin treatment indicated that YAP overexpression increased vimentin levels and decreased those of E-cadherin, with the opposite effects observed upon YAP silencing. However, metformin treatment decreased the effects of YAP overexpression and augmented the effects of YAP silencing (Fig. 6h). Furthermore, recent evidences suggest that metformin inhibits human cancer growth by inducing apoptosis via...
the mitochondria-mediated pathway [40–42] and control of mitochondrial structure and function is regulated by the YAP oncogenic pathway [43,44]. Basing on these evidences and results mentioned above, we hypothesized that metformin affects mitochondrial structure and function via inhibiting YAP and then restrains human cancer growth. Owing to metformin interferes with YAP-mediated NSCLC initiation, progression, and metastasis (Fig. 6), therefore YAP-knockdown can be used as a substitute for metformin to explore the hypothesis. Our results showed that YAP-knockdown and metformin indeed affects the integrity and function of mitochondria and induces the mitochondrial apoptosis analyzed by the proteins of mitochondrial cytC, cytosolic cytC, Bcl-2, Bcl-XL, Bax, PARP in A549 cells (Supplementary Fig. 7a–d). The mitochondrial apoptotic pathway (Supplementary Fig. 7d) and ROS level (Supplementary Fig. 7e) significantly increased after metformin treatment with this effect reversed by Yap overexpression. These results indicated that metformin affects mitochondrial structure and function via inhibiting YAP. Additionally, 3-Carboxy proxyl nitroxide (Mito-CP), the mitochondria-targeted compounds [38], disrupts mitochondrial membrane potential, structure and function (Supplementary Fig. 7f) and were co-treated with YAP-overexpression to investigate whether YAP influences mitochondrial apoptosis-mediated lung cancer cell growth and apoptosis. We found that combination treatment with Mito-CP and YAP-overexpression significantly reversed the ROS level and cell growth relative to that observed following individual treatment with Mito-CP (Supplementary Fig. 7f–h). We then explored apoptosis levels and markers by immunoblot of cleaved Caspase-3 and immunofluorescent staining of Fig. 7e) significantly increased after metformin treatment with this effect reversed by Yap overexpression. These results indicated that metformin affects mitochondrial structure and function via inhibiting YAP. Additionally, 3-Carboxy proxyl nitroxide (Mito-CP), the mitochondria-targeted compounds [38], disrupts mitochondrial membrane potential, structure and function (Supplementary Fig. 7f) and were co-treated with YAP-overexpression to investigate whether YAP influences mitochondrial apoptosis-mediated lung cancer cell growth and apoptosis. We found that combination treatment with Mito-CP and YAP-overexpression significantly reversed the ROS level and cell growth relative to that observed following individual treatment with Mito-CP (Supplementary Fig. 7f–h). We then explored apoptosis levels and markers by immunoblot of cleaved Caspase-3 and immunofluorescent staining of
Annexin V, finding that co-treatment with Mito-CP and YAP- overexpression resulted in lower levels of apoptosis than individual treatment with Mito-CP (Supplementary Fig. 7i, j). These data suggested that Yap knockdown involved in the transformation of mitochondrial structure and function to inhibit lung cancer cell growth and promote apoptosis. Collectively, our data indicated that the biological effects of metformin are due, at least in part, to the downregulation of YAP.

3.7. Verteporfin synergistically augments the effects of metformin on YAP function in lung cancer cells

Verteporfin (VP) inhibits YAP activity by disrupting the interaction between YAP and TEAD and represses the expression of YAP target genes [45,46]. Because both metformin and VP regulate YAP activity, we investigated whether the combination of these two drugs could exert a synergistic effect in NSCLC cells by examining the expression of the two YAP target genes (CTGF and Cyr61). Incubation with VP reduced the viability of A549 and H1299 cells in dose- and time-dependent manners, with the IC50 of VP 7.8 μM, 3.5 μM, and 1.5 μM for 24 h, 48 h, and 72 h, respectively, whereas that of metformin was 17.4 mM, 12.3 mM, and 7.8 mM, respectively, in A549 cells (Fig. 7a and b). In H1299 cells, these values were 8.0 μM, 3.7 μM, and 1.8 μM for VP and 18.4 mM, 12.1 mM, and 8.4 mM for metformin for the same respective time periods (Fig. 7a and b). Therefore, for subsequent experiments assessing the synergistic effects of combination treatment, we used 3.5 μM VP and 10 mM metformin in A549 cells and 4 μM VP and 10 mM metformin in H1299 cells for 48 h. The results showed that the combined index (CI) of metformin and VP was 0.532 in A549 cells and 0.502 in H1299 cells, indicating a synergistic effect in lung cancer cells (Fig. 7c). We then evaluated this effect on Yap activity in lung cancer cells, revealing that combination treatment with metformin and VP dramatically reduced the expression of CTGF and Cyr61 as compared with treatment with either metformin or VP in A549 cells (Fig. 7d). The synergistic effect of the two drugs was also shown on YAP target genes, CTGF and Cyr61, by immunofluorescence (Fig. 7e and Supplementary Fig. 8a, b). However, YAP expression was only decreased by treatment with metformin, suggesting that metformin reduced YAP transcription, whereas VP interfered with the interaction between YAP and TEADs to
reduce YAP target genes expression, which is represented function of YAP at protein level (Supplementary Fig. 8c). A549 cell growth in response to metformin and/or VP treatment was examined by CCK8, MTT, and colony formation assays. As shown in Fig. 7f, combination treatment with metformin and VP significantly reduced cell growth relative to that observed following treatment with either metformin or VP. We obtained similar results upon Ki67 staining (Supplementary Fig. 8d), the protein of Vimentin and E-cadherin (Supplementary Fig. 8e), colony formation (Fig. 7g), cell migration (Supplementary Fig. 8f) and cell invasion (Fig. 7h) assays. We then explored apoptosis levels and markers by immunoblot and flow cytometry, finding that co-treatment with metformin and VP resulted in higher levels of apoptosis than individual treatment with either drug (Fig. 7i and j). These results suggested that combination treatment with metformin and VP synergistically inhibited cell proliferation, promoted apoptosis, and suppressed lung cancer-cell migration/invasion by downregulating YAP.

3.8. Metformin inhibits the in vivo growth of in situ pulmonary xenografts

To address the central question regarding whether metformin treatment can delay tumorigenesis in lung cancer, we explored the in vivo antitumor activity of metformin in tumor xenografts assays. Approximately 2 weeks after subcutaneous injection of A549 cells into the concave niche of the cecum of experimental mice, larger tumors were observed in the control group (treated with phosphate-buffered saline) than in the experimental group (treated with metformin) (Fig. 8a and b), and we did not observe any significant change in the body weight of the animals in the two groups (Fig. 8a). The group treated with metformin exhibited increased survival (Fig. 8c), and semi-quantitative immunohistochemical analysis of Ki-67 (Fig. 8e), YAP (Fig. 8f), CTGF (Fig. 8g), Cyr61 (Fig. 8h), and vimentin (Fig. 8i) expression in the xenografts revealed that metformin treatment led to lower levels of these proteins as compared with those in the control group (Fig. 8a; n = 15), with opposite results observed for E-cadherin (Fig. 8d and j). These results indicated that metformin decreased levels of YAP and inhibited the in vivo growth of pulmonary xenografts. Moreover, we confirmed whether co-treatment with metformin and VP synergistically inhibited tumor growth and invasion by downregulating YAP in vivo. Our data showed that the body weight of YAP-overexpressing tumor loaded mice treated with combinations of metformin and VP was higher relative to that in mice receiving metformin treatment alone (Fig. 8k). The food intake every 3 days was subsequently increased in the group receiving co-treatment with metformin and VP relative to that given to the group treated with metformin alone (Supplementary Fig. 9a). These data indicated that the combined use of metformin and VP reduced metformin-specific side effects and possessed the synergistic effects of metformin in vivo. Furthermore, we observed that tumor sizes decreased significantly, and mouse survival increased significantly in the co-treatment group relative to the outcomes observed in groups receiving single treatment and the control group harboring YAP-expressing tumors (Fig. 8l and m). Additionally, we observed that YAP’s target genes’ CTGF and Cyr61mRNA and protein expression was significantly decreased in xenograft tumor tissue receiving co-treatment relative to that in groups receiving single treatment by analysis of RT-PCR (Supplementary Fig. 9b), RT-qPCR (Supplementary Fig. 9c, d) and Western blot (Supplementary Fig. 9e) assay. Likewise, the similar result of EMT marker protein vimentin but opposite result of E-cadherin was obtained in vivo by analysis of RT-qPCR (Supplementary Fig. 9f). These data suggested that co-treatment with metformin and VP synergistically inhibited tumor growth and invasion related to YAP in vivo and in vitro.

4. Discussion

In this study, we showed that YAP is upregulated and activated in NSCLC lung tissues and cell lines. Additionally, YAP knockdown inhibited lung cancer-cell proliferation, migration, and invasion and promoted apoptosis and senescence. Interestingly, metformin treatment induced similar effects on these biological processes. Therefore, we speculated that YAP inhibition might be an effect of metformin. As hypothesized, metformin treatment inhibited YAP expression and induced its phosphorylation, thereby reducing its nuclear translocation. Moreover, ectopic expression of YAP diminished the effect of metformin, whereas YAP silencing augmented it, indicating that YAP is a metformin target. We further confirmed the link between YAP and metformin in xenograft studies in nude mice, findings that co-treatment with metformin and VP, a YAP inhibitor, synergistically modulated YAP-specific biological processes. Collectively, our results suggested that YAP is involved in metformin-mediated biological processes (Fig. 8n).

Verteporfin (VP), a YAP specific inhibitor, can block the interaction between transcriptional coactivator YAP and transcriptional factor TEAD to repress YAP’s function [45,46]. During the past few years, several reports showed that VP is able to restrain cancer cell growth in some tumors, including retinoblastoma, endometrial and ovarian cancers [47–49]. However, the function and mechanism of VP inhibiting YAP activity in lung cancer were not yet clearly addressed. Our results showed that VP inhibited lung cancer cell growth, migration, invasion, EMT and induced cellular senescence and apoptosis meanwhile still had the ability to synergistically promote these effects of metformin on YAP function in lung cancer cells. The reason for the synergistic effect is that metformin decreases the YAP mRNA at transcriptional level, which then down regulates its target genes, Cyr61 and CTGF (Fig. 3). Of note, VP inhibits YAP function through restraining the interaction YAP and TEAD and then inhibits the expression of these YAP’s target genes, that is represented function of YAP at protein level (Fig. 7). Therefore, the co-treatment with metformin and VP acted synergistically in the modulation of the biological processes mentioned above with reducing side effects (Fig. 8). Collectively, basing on the function and mechanism mentioned above of cotreatment of metformin and VP, we believe that cotreatment of these two drugs can better cure human lung cancers and hopefully, that will be applied to treat other human cancers.

There are two mechanisms associated with the anticancer effects of metformin: metformin lowers circulating insulin, which can bind to the insulin receptor highly expressed in cancer cells, thereby indirectly decreasing cell proliferation; and metformin directly activates AMPK and subsequently inhibits mammalian target of metformin, leading to reduced cancer-cell proliferation [47–49]. Recent studies demonstrated that AMPK activation induces YAP phosphorylation and inhibits YAP transcriptional activity [36]. Additionally, LATSI/2 can be activated by an AMPK-dependent pathway to suppress YAP activity by phosphorylating YAP at S127 [37]. Specifically, AMPK phosphorylates angiomotin, which is associated with YAP, and increases its own stability, thereby leading to retention of YAP in the cytoplasm and promoting its phosphorylation by LATSI/2 [38]. AMPK can also directly phosphorylate YAP at S94 and inhibit YAP transcriptional activity by disrupting its interaction with TEAD. Moreover, AMPK directly phosphorylates YAP S61, reducing the expression of YAP target genes through an unknown mechanism [38]. Therefore, metformin might induce YAP phosphorylation to suppress its activity partly through an AMPK-dependent pathway. The effect of metformin could also be mediated independent of AMPK. A recent report showed that YAP is O-GlcNAcylated by an O-GlcNac transferase, and that this modification prevents the interplay between YAP and LATSI, and leads to reduced YAP phosphorylation and enhanced transcriptional activity [39]. Accordingly, metformin might exert its effect by suppressing glucose-induced YAP O-GlcNAcylation [50]. Consistently, we found that metformin treatment induced YAP phosphorylation at S127 along with its nuclear exclusion. The expression of two YAP target genes, CTGF and Cyr61, was also reduced, further confirming that metformin suppressed YAP transcription functions. Furthermore, we found that metformin repressed YAP mRNA and protein expression, with downregulation of YAP protein expression partially attributed to the binding of phosphorylated YAP (S127) to 14-

GlcNAcylation [50]. Consistently, we found that metformin treatment inhibited YAP transcription function by downregulating YAP.
3-3, leading to its cytoplasmic retention, and subsequent degradation by the ubiquitin–proteasome pathway. Our study confirmed that metformin decreased YAP luciferase activity, indicating that metformin downregulates YAP at the mRNA level. We hypothesized that metformin might be involved in the regulation of YAP transcription. To address our hypothesis, we isolated the core region of the YAP promoter. IRF-1 is important for the proliferation of human cancers, and IRF-1 silencing inhibits the growth of liver cancer cells. Additionally, IRF-1 mediates inflammation and increases pulmonary cathepsin S production [51]. Based on bioinformatics analyses, we found that the transcription factor IRF-1 might be involved in the attenuated YAP promoter activity mediated by metformin treatment. In order to further explore the mechanism of metformin attenuates YAP promoter activity by interfering with IRF-1 binding, we performed the UV-visible absorption spectral measurements and fluorescence polarization–based peptide displacement experiments, which are extracellular binding assays. Our result showed that metformin directly interfered with IRF-1 binding of YAP promoter not requiring other cell molecules (Fig. 4d, e and Supplementary Fig. 3m). Notably, our data showed that metformin depressed YAP promoter activity by interfering with the binding of IRF-1 and reducing YAP transcription. Our data showed that IRF-1 was able to induce YAP expression and that of its target genes, CTGF and Cyr61, after which upregulated YAP could then promote NSCLC initiation, progression, and metastasis. Collectively, our findings are consistent with the notion that YAP is a key factor in carcinogenesis. Therefore, these results showed that metformin downregulates YAP by competing with the transcription factor IRF-1 for binding on the YAP promoter, thereby inhibiting the growth of lung cancer.

In this study, we reported a novel function of metformin in regulating YAP through the transcription factor IRF-1 in NSCLC. In our model, metformin downregulates YAP by competing with IRF-1 for binding the YAP promoter in NSCLC cells (Fig. 8n). Our findings provide novel insights into the mechanism of MST-YAP-signaling regulation mediated by metformin in lung carcinogenesis. Therapeutically, metformin might be useful in the treatment of lung cancer. Additionally, because of the synergy exhibited between metformin and VP co-treatment, the combined use of metformin and VP could potentially reduce the side effects caused by their single use (at higher concentrations) and improve the quality of life of patients with lung cancer.

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Author contributions

Dan, Jin and Jiwei, Guo designed the experiments. Dan Jin, Jiwei Guo, Deqiang Wang, Yan Wu, Yong Gao, Cuijing Shao, Xiaohong Wang, Xin Xu, Shuying Tan performed the work. Dan Jin and Jiwei, Guo analyzed the data and competed the figures. Jiwei, Guo wrote the manuscript.

Conflicts of interest

The authors declare no competing financial interests.

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