YAP Suppresses Lung Squamous Cell Carcinoma Progression via Deregulation of the DNp63–GPX2 Axis and ROS Accumulation

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Abstract

Lung squamous cell carcinoma (SCC), accounting for approximately 30% of non–small cell lung cancer, is often refractory to therapy. Screening a small-molecule library, we identified digi- toxin as a high potency compound for suppressing human lung SCC growth in vitro and in vivo. Mechanistic investigations revealed that digi- toxin attenuated YAP phosphorylation and promoted YAP nuclear sequestration. YAP activation led to excessive accumulation of reactive oxygen species (ROS) by downregulating the antioxidant enzyme GPX2 in a manner related to p63 blockade. In patient-derived xenograft models, digi- toxin treatment efficiently inhibited lung SCC progression in correlation with reduced expression of YAP. Collectively, our results highlight a novel tumor-suppressor function of YAP via downregulation of GPX2 and ROS accumulation, with potential implications to improve precision medicine of human lung SCC. Cancer Res; 77(21); 1–13. ©2017 AACR.

Introduction

Lung cancer is the major health burden worldwide with high incidence and mortality (1). On the basis of the clinical and histological criteria, lung cancer can be classified into small-cell lung cancer (SCLC) and non–small cell lung cancer (NSCLC; ref. 2), and the latter is the most common subtype of lung cancer with a grim prognosis (3). Lung squamous cell carcinoma (SCC) accounts for approximately 30% of NSCLC cases (2), which equates to about 400,000 patients worldwide annually (4). Lung SCC is a treatment-refractory malignancy, which remains as largely incurable. Unlike lung adenocarcinoma (ADC) patients, patients with lung SCC do not have common oncogenic drivers such as EGFR mutation or ALK fusion (5), and seldom respond to those targeted therapeutic regimens (6–8). Therefore, the identification of efficient therapeutics is of urgent need for improvement of human SCC management.

The Hippo pathway is a newly identified pathway, which is originally known to restrict organ size via balancing cell proliferation and apoptosis (9). Emerging evidences have also indicated the prominent role of the Hippo pathway in regulating tumorigenesis (10). YAP is one of major downstream executors of the Hippo pathway. The Hippo signaling controls YAP activation through the modulation of its protein level and/or subcellular distribution in a MST-LATS dependent manner (11). The upstream components of Hippo pathway such as MST or LATS are observed to be switched off by mutations or epigenetic silencing and the downstream effects of the Hippo pathway such as YAP are hyperactivated via gene amplification or overexpression in a wide spectrum of human epithelial cancers (12). Indeed, YAP is commonly considered as a proto-oncogene in a majority of epithelial cancers including lung cancer (13–16). Interestingly, other studies have also demonstrated the tumor-suppressive role of YAP in hematological cancer and breast cancer (17, 18). These data indicate a cell or tissue-context function of YAP in cancer malignant progression.

Lung ADC and SCC are two distinct lung cancer subtypes whereas the inactivation of LKB1 could progressively promotes the transdifferentiation of lung ADC to SCC in the mouse model, which is potentially associated with malignant progression and drug resistance (19, 20). Interestingly, our work from animal...
models have previously identified different levels of YAP activation in lung ADC and SCC and found that YAP activation could largely abolish this phenotypic transition (21), indicating that YAP might function differently in these two major subtypes of NSCLC. We and others have convincingly shown that YAP acts as a potential proto-oncogene in promoting lung ADC malignant progression using both human cell lines and mouse models (21, 22). In contrast, the exact role of YAP in SCC still remains largely unknown.

Here, we have performed a small-molecule compound screening in human lung SCC cells and identified digitoxin as the one with high inhibitory potency. Unexpectedly, we find that digitoxin increases YAP nuclear sequesteration and boosts YAP activity. YAP activation unexpectedly leads to excessive accumulation of intracellular reactive oxygen species (ROS) level through downregulation of the antioxidant enzyme GPX2 expression in DNp63-dependent manner. Using preclinical PDX models, we have further found the correlation between YAP level and digitoxin efficacy in suppression of lung SCC growth, which holds important implication for precision medicine.

Materials and Methods

Human lung SCC specimen collection

All the human lung SCC specimens were collected in Fudan University Shanghai Cancer Center from November 2007 to July 2010, with patient written consents and the approval from the Institute Research Ethics Committee. The patient studies were conducted in accordance with International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS) ethical guidelines. All tumor specimens were taken at the time of surgical resection. Sixty-six of SCC samples were used for immunohistochemistry analysis.

Cell culture, transfection, and lentiviral infection

Human lung SCC cell lines HTB-182, L78, CRL-5889 and human normal bronchus epithelia cells HNBE were purchased from ATCC and cultured in RPMI-1640 supplemented with 10% FBS. HEK-293T were cultured in DMEM containing 10% FBS. HEK-293T were seeded in 24-well plates. Luciferase reporter and the indicated plasmids were co-transfected. Luciferase activities were detected 48 hours after transfection using Dual-Luciferase Assay kit (Promega) following the manufacturer’s instructions. pRL-TK was co-transfected as internal control. Experiments were done in triplicates.

Cellular functional assay

Cell viability was measured by CellTiter-Glo luminescent cell viability assay (Promega). Briefly, 50 μL of assay reagent was added to the wells and mixed at room temperature. After 10 minutes, intracellular ATP content was measured by a luminescence multilabel counter.

Microarray analysis

Gene profiling were performed using Affymetrix Human Genome U1219 array Strip. The expression of genes was calculated and analyzed by Harmony 3.5 software.
normalized with robust multichip average. The gene expression data were subjected to pathway analysis using GSEA (version 2.2.10) from Broad Institute. Signal-to-noise ratio metric was used to discover differential expression genes and enrichment analysis was performed with Kyoto Encyclopedia of Genes and Genomes pathway source by permuting 1,000 times of genes sets. Significantly enriched pathways were scored and ranked by default non-P value.

**Immunohistochemistry**

IHC was performed as described previously (24). Paraffin-embedded mice tissues (lung, liver, and intestine) and xenograft tumors were incubated with the following antibodies: anti-YAP (Santa Cruz Biotechnology; 1:500 dilution), anti-DNp63 (Santa Cruz Biotechnology; 1:500 dilution), anti-Ki67 (Leica; 1:500 dilution), anti-cleaved caspase-3 (Cell Signaling Technology; 1:500 dilution), and anti–8-oxo-dGuo (Abcam; 1:500 dilution). The immunostaining was reviewed and scored blindly. The scoring system for grading level of nuclear YAP, nuclear p63 and 8-oxo-dGuo expressions was reported previously (21). Briefly, intensity was graded as 0–4 to indicate undetectable, weak, moderate, strong, and extremely strong staining. The expression level was scored by multiplying intensity by percentage of positive cells. The proliferation rate was evaluated by counting Ki67-positive nuclear staining. Cell death was assessed by the analysis of cleaved caspase-3 staining. Cellular oxidative stress was assessed by 8-oxo-dGuo staining. To calculate cells positive for Ki67, cleaved caspase-3 and 8-oxo-dGuo, at least 30 high-power fields were evaluated for each group.

**Immunoblotting**

Cells were lysed in lysis buffer and subjected to Western blot analysis with the following primary antibodies: anti-YAP (Santa Cruz Biotechnology, 1:500 dilution), anti–phospho-YAPSer127 (Cell Signaling Technology, 1:500 dilution), anti-GPX2 (Abcam, 1:500 dilution), anti-DNp63 (Santa Cruz Biotechnology, 1:500 dilution), anti-LATS1 (Cell Signaling Technology, 1:1,000 dilution), anti-MST1 (Cell Signaling Technology, 1:1,000 dilution), anti-histone H3 (Proteintech, 1:1,000) and anti-ACTIN (Sigma, 1:5,000 dilution).

**Nuclear and cytoplasmic protein extraction**

Preparation of nuclear and cytoplasmic extract was performed as previously described using Beyotime Nuclear and Cytoplasmic Protein Extraction Kit (21). Briefly, Cells were harvested by adding 0.125% trypsin-EDTA and lysed in buffer A on ice for 15 minutes and centrifuged at 16,000 × g for 5 minutes. The supernatant containing the cytoplasmic proteins was stored for use. The pellet was resuspended in buffer B. After incubation on ice for 30 minutes, lysates were centrifuged at 16,000 × g for 15 minutes and the supernatants containing the nuclear proteins were stored for use.

**Intracellular ROS detection**

Intracellular ROS detection was performed following manufacturer’s instructions (Beyotime Reactive Oxygen Species Assay Kit). Briefly, cells of indicated groups were seeded in 6-well plate. Forty-eight hours later, cells were incubated with DCFH-DA probe for 20 minutes. Then the cells were harvested and subjected to FACS analysis.

**Mice treatment and histopathological analysis**

All mice were housed in a specific pathogen-free environment at the Shanghai Institute of Biochemistry and Cell Biology and treated with strict accordance with protocols approved by the Institutional Animal Care and Use Committee of the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences.

HTB-182 cells (10⁶ cells) and patient derived lung SCC xenografts were subcutaneously transplanted into nude mice (n = 3 for xenograft assay and n = 4–6 for PDX model each group). When the average tumor volumes reached about 200 mm³, nude mice were administrated with digitoxin (1 mg/Kg) or vehicle via intraperitoneal injection. Tumor volume and nude mice weight were measured every other day. Wild-type mice were also treated with digitoxin (1 mg/Kg) for 3 weeks. All mice were then sacrificed for pathological examination and tumors were dissected for molecular analysis. Histopathological analysis was conducted as previously described. Briefly, mice tissues including lung, liver, intestine, and xenograft tumors were fixed in 4% formalin, embedded in paraffin and sectioned for hematoxylin and eosin staining.

**Plasmid construction**

To construct the GPX2 expression plasmid, the coding sequence of GPX2 was amplified with primers: GPX2-Forward: ACCAATTCGCAACATCGAGAAACAACTCA-TCTCAGAAGAGGATCTGGCTCACTCTGCGCTTCACCATGGCTT; GPX2-Reverse: CGGATCCCTATATGGCAACTTTAAGGAGG. The DNA fragment was then inserted into pCDH vector. The GPX2 2074-bp promoter fragment was amplified with following primers:

forward: CGGGCTAGCTCTAGAAAAATTCCT-ACAAACAA;
reverse: CCCCTGAGGGTGAGGGGCAAATTTGCCAGTGAGGCCCCGC.

The DNA fragment was then inserted into the pGL3-Basic vector. The human YAP expression vector is a gift from Professor Kunliang Guan. The human YAP shRNA construct is generously given by Professor Zengqiang Yuan. The human LATS1 and MST1 shRNA construct is kindly given by Professor Bin Zhao. YAP expression vectors, including YAP S127A, YAP 5SA and YAP 5SA-DeIC were generated as previously described (21). The plasmids pCDH-YAP S94A and pCDH-YAP WW domain mutant were constructed using the QuikChangeXL Site-Directed Mutagenesis Kit (Agilent Technologies).

**Statistical analysis**

Statistical analyses were performed by the Student t test (two-tailed) using Prism GraphPad software. Error bars represent SEM (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

**Results**

**Identification of digitoxin as a top hit in suppression of lung SCC growth**

To identify efficient lung SCC growth inhibitors, we screened a chemical library of 465 compounds targeting various biological processes in human lung SCC cell line L78. We identified 10 candidates with most significant effects upon cell viability and cell death (Fig. 1A–C). Most of these compounds are known as anti-cancer drugs, suggesting the feasibility of our screen system. Interestingly, digitoxin and digoxin, which share similar chemical structure, stand out as top
Figure 1.
Identification of digitoxin as a compound efficiently suppressing lung SCC growth in vitro and in vivo. A and B, Plots showing cell death (A) and growth inhibition (B) of lung SCC L78 cells treated with a library of small-molecule compounds at 1 μmol/L compared with the control. Digitoxin and digoxin are highlighted in red. C, Top 10 candidates with most significant effects upon cell death and growth inhibition. D, Relative cell viability of lung SCC cell lines L78, HTB-182, CRL-5889, and human normal bronchial epithelial cells HNBE treated with indicated concentration of digitoxin for 72 hours compared with vehicle control. E, HTB-182 and CRL-5889 cells treated with digitoxin (100 nmol/L) in soft agar colony formation assay. F and G, Digitoxin treatment in xenograft assay of HTB-182 cells (n = 3 for each group). Tumor volume (F) and weight (G) are shown. H and I, Representative photos of Ki67 (H) and cleaved caspase-3 (CC3; I) immunostaining in HTB-182 xenograft tumors treated with digitoxin. Statistical analyses of the Ki67 and CC3-positive index are also shown. ***, *P < 0.01; ***, *P < 0.001.
Digitoxin is a cardiac glycoside widely used in clinical practice for treating heart failure and atrial fibrillation, potentially through inhibition of the sodium–potassium adenosine triphosphates' (Na⁺/K⁺-ATPase) complex to increase the intracellular calcium level (25). We next tested the efficacy of digitoxin on other human lung squamous cell lines (HTB-182, L78 and CRL-5889) as well as human normal bronchial epithelial cells HNBE. Compared with HNBE cells, the lung SCC cell lines showed higher sensitivity to digitoxin (Fig. 1D). Consistently, digitoxin treatment almost completely abolished the colony formation ability of HTB-182 and CRL-5889 cells (Fig. 1E).

We further found that digitoxin treatment efficiently inhibited human SCC growth in xenograft assay, indicated by the significantly reduced tumor growth and tumor weights (Fig. 1F–G). Consistently, a lower proliferation rate indicated by Ki-67 staining and a higher apoptosis rate indicated by cleaved caspase-3 staining were observed in tumors with digitoxin treatment (Fig. 1H–I). Taken together, these data indicated that digitoxin worked efficiently in suppressing lung SCC growth both in vitro and in vivo.

Digitoxin enhances YAP activity in lung SCC

Previous study has showed that digitoxin gravitates to the WW domain of YAP in a manner similar to the binding of canonical PPxY ligands (26, 27). This led us to investigate if digitoxin affects YAP activity. Interestingly, we found that digitoxin treatment indeed attenuated YAP-S127 phosphorylation (Fig. 2A) and promoted YAP nuclear retention (Fig. 2B and C). Consistently, we observed a significant increase of YAP nuclear staining in tumors treated with digitoxin (Fig. 2D). Together, these data indicated that digitoxin treatment elevated YAP activity in human SCC cells through currently unknown mechanism.

Figure 2.

Digitoxin promotes the nucleus translocation of YAP. A, Western blot detection of endogenous YAP and phosphorylated YAP (S127) levels in L78 and HTB-182 cells treated with digitoxin. Actin served as control. B, Immunofluorescence staining of L78 and HTB-182 cells treated with digitoxin. YAP (green) and nuclei (blue). C, Western blot detection of nuclear and cytoplasmic YAP levels in CRL-5889 and HTB-182 cells treated with digitoxin. Histone H3 served as nuclear control and tubulin served as cytoplasmic control. D, Representative photos for YAP immunostaining and statistical analysis in HTB-182 xenograft tumors with or without digitoxin treatment. ***. P < 0.001.
Modulation of the MST1/LAST1/YAP axis suppresses lung SCC cell growth

The cytostatic effect of digitoxin and its promotive role on YAP activity led us to speculate that digitoxin inhibits SCC cell proliferation through activation of YAP. We found that ectopic expression of YAP activating mutant (YAP-S127A) dramatically suppressed lung SCC cell proliferation and decreased the soft agar colony formation (Fig. 3A and B; Supplementary Fig. S1A). Moreover, we found that knockdown of either LATS1 or MST1, the upstream negative regulators of YAP in the canonical Hippo pathway (28), attenuated lung SCC cell proliferation and colony formation (Fig. 3C–F; Supplementary Fig. S1B–S1C).

We further found that the growth inhibitory function of YAP in lung SCC was impaired when its carboxyl-terminal trans-activation domain was deleted (Supplementary Fig. S1D–S1E), indicating that the transcriptional activity of YAP is indispensable for its function. Previous studies showed that TEADs and p73 are major transcriptional factors binding to YAP and execute different functions in a context-dependent manner (29, 30). Disruption of the interaction between YAP and TEADs by employing YAP S94A mutant (31, 32), but not disruption of the interaction between YAP and p73 by using YAP WW domain mutant (29), abolished the suppressive role of YAP on lung SCC cell proliferation, suggesting the function of YAP in lung SCC depended on its binding to TEADs (Supplementary Fig. S1F–S1G). Together, these data indicated that YAP suppressed SCC cell growth potentially through the transcriptional activation of the YAP–TEAD complex.

YAP suppresses lung SCC growth through disruption of ROS homeostasis

To further explore the underlying mechanism of YAP in regulating lung SCC cell growth, we performed gene-expression microarray profiling and gene set enrichment...
analysis (GSEA) to evaluate the global transcriptomic changes associated with YAP activation. Interestingly, we found that gene sets of peroxisome pathway and glutathione metabolism were significantly enriched in SCC cells with YAP-S127A overexpression (Fig. 4A). Real-time PCR quantification further confirmed that YAP activation decreased the expression of multiple signature genes for ROS clearance (Fig. 4B). Consistently, we observed the increased cytosolic ROS accumulation in L78, HTB-182, and CRL-5889 cells with and without ectopic YAP S127A expression. Downregulation (%) was compared with vector control. C and D, ROS levels indicated by DCFH-DA staining in L78 (C) and CRL-5889 (D) cells with and without ectopic YAP S127A expression. E, Relative cell growth of L78, CRL-5889, and HTB-182 cells with or without ectopic YAP S127A expression with or without NAC (5 mmol/L) treatment. *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 4.

YAP activation promotes ROS accumulation. A, Significantly enriched gene sets in human lung SCC cells with or without ectopic YAP S127A expression. GSEA plots of the peroxisome and glutathione metabolism pathways are shown. B, Real-time PCR quantification of signature genes in peroxisome and glutathione metabolism pathways in L78, HTB-182, and CRL-5889 cells with and without ectopic YAP S127A expression. Downregulation (%) was compared with vector control. C and D, ROS levels indicated by DCFH-DA staining in L78 (C) and CRL-5889 (D) cells with and without ectopic YAP S127A expression. E, Relative cell growth of L78, CRL-5889, and HTB-182 cells with or without ectopic YAP S127A expression with or without NAC (5 mmol/L) treatment. *P < 0.05; **P < 0.01; ***P < 0.001.

The P63/GPX2 axis mediates the inhibitory function of YAP

To identify potential downstream executors of YAP in regulating ROS homeostasis, we examined the transcriptional level of multiple ROS scavenging genes. Most of those genes are down-regulated upon YAP activation in lung SCC cells (Fig. 4B). Notably, GPX2, which encodes the antioxidant enzyme glutathione peroxidase 2, was most significantly and consistently downregulated in L78, HTB-182 and CRL-5889 cells. Furthermore, we found that ectopic YAP S127A expression significantly decreased GPX2 expression at both mRNA
Figure 5.

YAP promotes ROS accumulation via the DNp63–GPX2 axis. **A** and **B**, GPX2 mRNA (**A**) and protein (**B**) levels in HTB-182 and L78 cells with or without ectopic YAP S127A overexpression. **C** and **D**, GPX2 mRNA (C) and protein (D) level in HTB-182 and L78 cells with or without YAP knockdown. **E**, GPX2 promoter reporter assay with or without DNp63 overexpression in HEK-293T cells. **F** and **G**, GPX2 mRNA in HTB-182 (**F**) and L78 (**G**) cells with or without YAP S127A and/or DNp63 expression. **H** and **I**, GPX2 protein level in HTB-182 (**H**) and L78 (**I**) cells with or without YAP S127A and/or GPX2 expression. **J**, Relative cell growth of HTB-182 and L78 cells with or without YAP S127A and/or GPX2 expression. ****, $P < 0.01$; ***$, P < 0.001.$
and protein level (Fig. 5A and B). Conversely, YAP knockdown upregulated GPX2 expression (Fig. 5C and D). We have previously shown that YAP decreased DNp63 expression by upregulating the transcriptional repressor ZEB2 (21). Consistently, we find a negative correlation between YAP and P63 levels in human SCC samples (Supplementary Fig. S2A–S2B). Interestingly, GPX2 was reported to be a direct target of DNp63 (33). Consistent with this, our data showed that DNp63 over-expression significantly enhanced the GPX2-promoter activity (Fig. 5E). Moreover, the downregulation of GPX2 level by YAP activation could be rescued by DNp63 co-expression (Fig. 5F–I). These data demonstrated that YAP downregulated the expression of the antioxidant enzyme GPX2 in a DNp63-dependent manner. We next asked if GPX2 indeed mediated the inhibitory function of YAP in SCC cells. Activation of YAP reduced GPX2 expression and suppressed SCC cell proliferation whereas reintroduction of GPX2 rescued the inhibitory function of YAP (Fig. 5J; Supplementary Fig. S3A–S3B).

Boosting YAP activity by digitoxin treatment decreased DNp63 level and in turn suppressed GPX2 expression (Fig. 6A and B; Supplementary Fig. S3C). Moreover, digitoxin treatment significantly promoted excessive ROS accumulation (Fig. 6D). Together, our data demonstrated that re-activation of YAP by genetic manipulation or digitoxin treatment disrupted ROS homeostasis via the DNp63–GPX2 signaling axis.

Reactivation of YAP through digitoxin treatment efficiently suppresses the growth of lung SCC with low YAP expression in PDX model

Patient-derived xenograft (PDX) model serves as a useful platform for evaluating the therapeutic efficacy. To ask whether there exists the potential link between digitoxin efficacy and YAP nuclear level, we performed digitoxin treatments in two SCC PDX models with relative low or high nuclear YAP level (Fig. 7A and B). No obvious loss of body weights was found in mice with digitoxin treatment (Supplementary Fig. S4A–S4B).

Although digitoxin treatment increased YAP nuclear level in intestine and to a much less extent in liver and lung, these organs were pathologically normal and displayed no change of cell proliferation and apoptosis rates, which were indicated by Ki67 and cleaved caspase 3 IHC staining respectively (Supplementary Fig. S4C).

Interestingly, we found that the SCC model with low YAP level (PDX#1) was much more sensitive to digitoxin treatment: digitoxin treatment significantly suppressed the tumor growth (Fig. 7C). In contrast, the SCC PDX model with high YAP expression (PDX#2) was refractory to digitoxin treatment (Fig. 7D). Significant increase of YAP nuclear
Figure 7.
The antitumor efficacy of digitoxin in PDX mouse models correlates with low nuclear YAP level. A and B, Representative photos for YAP immunostaining in PDX#1 (A) with low nuclear YAP level and PDX#2 (B) with high nuclear YAP level. C and D, Tumors growth of lung PDX#1 (C) and PDX#2 (D) with or without digitoxin treatment. E and F, Representative photos for YAP (E) and 8-oxo-dGuo (F) immunostaining and statistical analysis in lung SCC PDX#1 with or without digitoxin treatment. G and H, Representative photos for YAP (G) and 8-oxo-dGuo (H) immunostaining and statistical analysis in lung SCC PDX#2 with or without digitoxin treatment. **, P < 0.01; ***, P < 0.001; ns, nonsignificant.
staining and ROS accumulate (indicated by 8-oxo-dGuo staining) were observed in the PDX#1 with digitoxin treatment (Fig. 7E and F). However, the YAP<sup>hi</sup> SCC PDX#2 with digitoxin treatment showed no significant changes in YAP nuclear expression and ROS accumulation (Fig. 7G and H). These data collectively demonstrated that reactivation of YAP specifically suppressed the growth of lung SCC PDX with low nuclear YAP level.

**Discussion**

In this study, we have revealed an unexpected role of YAP in the suppression of lung SCC growth through disruption of intracellular ROS homeostasis via the DNp63–GPX2 axis. We have further identified digitoxin as a potential YAP activator and cytostatic agent for human lung SCC with low YAP nuclear level. Reactivation of YAP by digitoxin shows tumor growth inhibition potential in both human lung SCC cell xenograft and preclinical lung SCC PDX models. These data demonstrate that the novel tumor-suppressive function of YAP correlates with digitoxin treatment and provides potential therapeutic strategy for treatment of human lung SCC with low YAP expression.

The oncogenic property of YAP is well documented in lung cancer, especially in lung ADC. For example, we and others have shown that YAP activation is important for lung ADC malignant progression (16, 21, 22) and the response to EGFR-targeted therapy in lung ADC cells (35). However, the function of YAP in lung SCC still remains elusive. Previous studies show that the majority of human lung SCC exhibit low YAP levels (21, 36), but not lung ADC may explain why silencing DNp63 in lung SCC results in decreased cell proliferation and tumor growth, in stark contrast with the oncogenic role of YAP in lung ADC. Those data implicate the cell lineage–specific role of YAP in lung cancer development. YAP activation accelerates lung ADC progression through enhancing pro-survival and pro-metastatic genes expression (16, 22), whereas suppresses lung SCC growth through GPX2 downregulation. Our data also support an involvement of the canonical YAP–TEAD complex in lung SCC growth suppression other than previously reported YAP–P73 complex in myeloma (17). We further show that YAP reduces GPX2 expression through downregulating SCC lineage-specific transcription factor DNp63. The dominant role of DNp63 in lung SCC survival but not lung ADC may explain why silencing DNp63 in lung SCC by YAP activation leads to the inhibition of lung SCC growth.

The fascinating connection between YAP and metabolic program, including nutrient traits, energy status, and redox homeostasis begins to emerge in recent years. YAP transcriptional activity can regulate cellular response to glucose, for example, phosphofructokinase (PFK1), responsible for the first committed step of glycolysis, binds to TEADs and promotes their functional and biochemical cooperation with YAP (37). The central metabolic sensor AMP-activated protein kinase (AMPK) can inhibit YAP activation through either direct YAP phosphorylation or modulation of LATS1/2 activity via angiomotin-like 1 (AMOTL1; refs. 38, 39). The function of YAP in redox homeostasis is mainly studied in cardiomyocytes. YAP activation can prevent cardiomyocyte death during severe oxidative stress condition (40).

Similarly, activation of YAP can protect cardiomyocyte cell against ischemia/reperfusion (I/R) injury, potentially through upregulation of antioxidant genes CAT and MnSOD (41). In contrast, how YAP regulates redox homeostasis in cancer remains largely unknown. Our data demonstrate that YAP activation in SCC results in the redox reprogramming evidenced by elevated ROS accumulation. YAP promotes ROS accumulation through downregulation of those ROS scavenger genes such as GPX2, which might result in a severe defect of ROS clearance and trigger cell growth inhibition.

Most efforts have been made to identify YAP inhibitors given its oncogenic function in a majority of epithelial cancer (42–47). However, discovering YAP agonists is still in its infancy. Digitoxin, a clinical drug for heart failure treatment, was reported to have capability to manipulate several signaling pathways such as MAPK, SRC kinase, and PI3K signaling. Recently, several studies have also indicated that digitoxin and digoxin can inhibit the malignant progression through targeting the transcriptional activities of AP-1 and/or NF-kB in various cancer types, serving as a promising anti-cancer drug (48–50). Our results show the promising efficacy of digitoxin in boosting YAP activity although detailed biochemistry mechanisms need to be addressed. We anticipate that digitoxin will be a useful tool for further understanding the physiological and pathological role of YAP in a variety of additional experimental settings. Digitoxin exhibits potential efficacy in primary SCC tumors derived from patients with minimal toxicity. Nonetheless, a large scale of lung SCC PDX models are necessary to test the in vivo efficacy and toxicity of digitoxin in the future. Moreover, these conclusions are based on studies with growth of lung cancer cells subcutaneously, and future studies are needed to determine the effects of digitoxin upon tumors growth in the lung environment. Together, our present study provides a proof of principle that YAP could be a promising therapeutic target for those lung SCC with low YAP activity and YAP expression level might serve as the biomarker for precision medicine.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: H. Huang, W. Zhang, H. Chen, H. Ji Development of methodology: H. Huang, W. Zhang Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Zhang, Y. Gao, G. Hu Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Huang, W. Zhang, L. Deng, C. Li Writing, review, and/or revision of the manuscript: H. Huang, W. Zhang, H. Ji

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W. Zhang, Y. Pan, F. Li, X. Ma, S. Hou, J. Xu, P. Li, X. Li, H. Chen, L. Zhang, H. Ji Study supervision: H. Chen, H. Ji

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