Sestrin2 mediates FOXM1 expression to block the EMT process in non-small cell lung cancer through the AMPK/YAP pathway

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Non-small cell lung cancer (NSCLC) is characterized by high incidence and mortality, severely threatening human health. The infinite growth and metastasis of NSCLC cells result in a poor prognosis. Therefore, our study was to investigate the mechanism of Sestrin2 on the epithelial-mesenchymal transition (EMT) process of NSCLC cells. Human embryonic lung fibroblasts, NSCLC cell lines, and nude mice were experimental subjects in this study. qRT-PCR and western blot were performed to evaluate the mRNA and protein expression of genes. CCK-8 and EdU assay were conducted to detect cell proliferation. The scratch test and Transwell assay were applied to examine cell migration and invasion. The bioinformatics analysis and Co-IP assay were employed to predict and consolidate the interaction between YAP and TEAD. We found the expression of Sestrin2 was declined but the expression of YAP was elevated in NSCLC cells. Sestrin2 sufficiency or YAP silencing could effectively impair cell growth and metastasis. Mechanistically, YAP interacted with TEAD to enhance FOXM1 expression. Additionally, the elevation of FOXM1 abolished the inhibitory influences of Sestrin2 sufficiency on NSCLC cell growth, invasion, and EMT process. Eventually, Sestrin2 elevation attenuated tumor growth in mice via modulation of the AMPK/YAP/FOXM1 axis, which was reversed by FOXM1 overexpression. Our consequences suggested Sestrin2 could inhibit the activation of YAP via prompting AMPK phosphorylation and then suppress FOXM1 expression through the interplay between YAP and TEAD to impair the capacities of NSCLC cell proliferation, migration, invasion, and EMT. This study provided a novel mechanism of Sestrin2 in NSCLC.

Key words: Sestrin2; YAP; FOXM1; EMT; non-small cell lung cancer

Based on cancer statistic data published in 2020, there were 2,206,771 new cases and 1,796,144 death cases of lung cancer, accounting for about 11.4% and 18.0% of all cancer cases and ranking second and first among all cancer types, respectively [1]. As a major subtype of lung cancer, non-small cell lung cancer (NSCLC) accounts for around 85% of lung cancer [2]. At present, surgical resection, radiotherapy, and chemotherapy have significantly improved the 5-year survival rate of NSCLC patients [3]. However, due to the recurrence and metastasis of lung cancer cells, the prognosis of NSCLC patients is still less than satisfactory [4]. Therefore, seeking crucial molecules for cancer cell metastasis in NSCLC patients deserves further investigation.

Sestrin2 is one of the elements of the Sestrin family proteins (Sestrin1, Sestrin2, Sestrin3) which are featured with multi-biological function [5]. As previously reported, Sestrin2 is closely correlated with tumor progression [6, 7].

For instance, Sestrin2 was lowly expressed and its overexpression could attenuate stemness in colorectal cancer by mediating the Wnt/β-catenin pathway [7]. Chen et al. proposed Sestrin2 was downregulated in NSCLC tissues and was conducive to the prognosis of NSCLC patients [8], indicating the momentous role of Sestrin2 in NSCLC. However, another study reported that Sestrin2 expression was higher in lung cancer cells than in normal lung cells and knockdown of Sestrin2 suppressed cell proliferation, migration, sphere formation, and drug resistance in a non-small cell lung cancer cell line (A549 cells) [9]. Currently, there are few studies on Sestrin2 in NSCLC. Whether Sestrin2 is a cancer-promoting molecule or a cancer suppressor molecule in NSCLC, and what is the molecular regulatory mechanism behind it? All of them need to be further discussed.

Yes-associated protein (YAP) is a transcriptional co-activator serving as a Hippo signaling effector [10].

Massive evidence reported YAP was implicated in various types of cancers such as cervical cancer, gastric cancer, and NSCLC [11–13]. In addition, YAP was involved in affecting multiple tumor activities including cell migration, invasion, epithelial-mesenchymal transition (EMT) process, stemness [14, 15]. A previous study revealed Sestrin2 could suppress YAP activation to regulate cell proliferation and aggravate corneal epithelial damage [16]. Bian et al. also demonstrated that Sestrin2 suppressed YAP activation to improve renal damage by regulating the Hippo pathway in diabetic nephropathy [17]. Based on the above description, we reasonably conjectured that YAP activation might be implicated in Sestrin2-mediated functions in NSCLC. Additionally, FOXM1 has been wildly reported in tumor fields and identified as a cancerogenic gene [18–20]. In NSCLC, FOXM1 was essential to tumorigenesis and contributed to poor clinical prognosis [19]. As previously described, YAP/FOXM1 axis was closely related to EMT-associated EGFR inhibitor resistance in NSCLC [21], indicating YAP might interact with FOXM1 to affect the malignancy of NSCLC. Therefore, in this study, whether Sestrin2 is by regulating YAP-FOXM1 to exert functions on the progression of NSCLC deserves to further be explored.

In this study, we evidenced our hypothesis at the cellular and animal levels and found that Sestrin2 restrained the activation of YAP protein via regulating the AMPK pathway, then further affecting FOXM1 expression through YAP-TEAD interaction and ultimately suppressing NSCLC cell proliferation, migration, invasion, and EMT process. Our findings might provide novel therapeutic targets for NSCLC treatments.

Materials and methods

Cell culture. Human embryonic lung fibroblast cells (MRC-5) and NSCLC cell lines (including A549, NCI-H1299, CALU-3, and 95-D) cells were acquired from ATCC (Manassas, VA, USA). MRC-5 cells were cultured in Minimum Essential Medium (MEM, Thermo Fisher Scientific, USA) and Calu-3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific). Other cell lines including A549, NCI-H1299, CALU-3, and 95-D cells were cultured in RPMI-1640 (Thermo Fisher Scientific). All mediums were supplemented with 10% FBS (Gibco, USA) and compound antibiotics (Beyotime, China). All cells were cultured in an incubator of 5% CO_2 flow at 37°C.

Cell transfection. The overexpression vector of Sestrin2 (oe-Sestrin2), the short hairpin RNA targeting YAP (sh-YAP), the overexpression vector of TEAD (oe-TEAD), the overexpression vector of FOXM1 (oe-FOXM1), and their negative control groups (pcDNA3.1, sh-NC) were customized from GenePharma (Shanghai, China). For *in vitro* transfection, NCI-H1299 cells seeded on 6-well plates were transfected with oe-Sestrin2, sh-YAP, oe-TEAD, oe-FOXM1, and their corresponding negative control groups using Lipofectamine™ 3000 (Invitrogen, CA, USA) according to the instruction. After 48 h, the transfected cells were used for relevant experiments.

RNA isolation, reverse transcription, and RT-qPCR. We employed qRT-PCR to examine the expression of Sestrin2, YAP, TEAD, and FOXM1 in the mentioned cells or lung tumors of mice. In brief, we first extracted the total RNA from samples using the TRIzol reagent (Invitrogen, USA). Then, Prime Script Reverse Transcription Reagent Kit (TaKaRa, China) was applied for cDNA synthesis. SYBR Premix Ex Taq II Kit (TaKaRa) was used for the qPCR process. Below are listed the primer sequences: Sestrin2 (F): 5'-GCATTACCTGCTGCTGCATA-3'; Sestrin2 (R): 5'-AAGGCCTGGATATGCTCCTT-3'; YAP (F): 5'-GCATGATCTGCCCTAAGGC-3'; YAP (R): 5'-TGACC-GCCGAGTACACCAT-3'; TEAD (F): 5'-TCTGGGCGGACT-TAAACTGC-3'; TEAD (R): 5'-GAACCTCGCATACTCC-GTCTCT-3'; FOXM1(F): 5'-CGTCGGCCACTGATTCT-CAAA-3'; FOXM1 (R): 5'-GGCAGGGGATCTCTTAGGTTC-3'; GAPDH (F): 5'-GGGAGCCAAAAGGGTCAT-3'; GAPDH (R): 5'-GAGTCCTTCCACGATACCAA-3'.

Finally, all original data were calculated by using the $2^{-\Delta\Delta Ct}$ formula. GAPDH served as the reference gene.

Western blot. We detected the protein levels of targeted molecules in the mentioned cells or in lung tumors from mice. Shortly, the samples were lysed in RIPA buffer (Beyotime) to obtain total proteins. Equivalent proteins were isolated using SDS-PAGE and were transferred onto PVDF membranes. Subsequently, the membranes underwent the immersion of non-fat milk for 15 min. Specific primary antibodies diluted following the corresponding instructions were performed for incubation of membranes overnight at 4°C. Next, an HRP-labeled secondary antibody (1:10000, Absin, #abs20039, China) incubated the membranes. ECL chemiluminescent reagent (Beyotime) was applied for the visualization of protein bands. The gray values were detected by using ImageJ (National Institutes of Health, USA). Notably, all primary antibodies were purchased from Abcam (UK) and the detailed information were as follows: anti-Sestrin2 (ab178518, 1:1000), anti-p-YAP (ab76252, 1:20000), anti-YAP (ab205270, 1:1000), anti-E-cadherin (ab1416, 1:50), anti-N-cadherin (ab245117, 1:1000), anti-Vimentin (ab137321, 1:2000), anti-Snail (ab216347, 1:1000), anti-Twist (ab175430, 1:1000), anti-p-AMPK (ab92701, 1:4000), anti-AMPK (ab32047, 1:2000), anti-TEAD (ab133533, 1:3000), anti-FOXM1 (ab207298, 1:1000), and anti-GAPDH (ab8245, 1:3000).

Cell counting kit-8 (CCK-8) assay. NCI-H1299 cells with indicated transfection were seeded onto 96-well plates. In the next day, 10 μl CCK-8 solution (Beyotime) was added to each well. After incubation for 2 h, the absorbance at 450 nm was detected by a microplate reader (Thermo Fisher Scientific).

5-ethynyl-2'-deoxyuridine (EdU) assay. BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 488 (Beyotime) was

applied to measure the cell proliferation of NCI-H1299 cells which were treated with indicated transfections. Following the instructions, cells were incubated with EdU medium diluent (Beyotime) for 3 h. After all cells were fixed and permeabilized, the reaction cocktail was applied to react with the EdU for 30 min. DAPI was used to stain cell nuclei. Fluorescence microscopy (Bio-Rad, USA) was provided to observe EdU-positive cells.

Scratch test. NCI-H1299 cells with indicated transfections were implanted on 6-well plates with cellular monolayers overnight. Cells were scraped with a micropipette tip and recorded the width between the scratches. After the cells were washed with PBS solution, cells continued to culture for 48 h and then recorded the width again. The widths evaluated the ability of migration.

Transwell assay. A 24-well Transwell insert system (Corning, USA) was applied for the detection of migratory and invasive abilities of NCI-H1299 cells. For cell migration assay, NCI-H1299 cells (1×10^6 cells/ml) were inoculated on the upper chamber which contained FBS-free RPMI-1640. The lower chamber was maintained with RPMI-1640 including 10% FBS. After 24 h, 95% alcohol fixed the migrating cells on the below side of the chamber. 1% crystal violet (Sigma-Aldrich, USA) was applied to stain the migrated cells for 5 min. Finally, the stained cells were observed under an inverted microscope (Olympus, Japan).

For cell invasion assay, the upper Transwell chamber was filled with Matrigel (Becton, USA). Other processes were as same as in the migration assay.

Co-immunoprecipitation (Co-IP) assay. A Co-IP assay was conducted for verifying the interaction between YAP and TEAD. Shortly, Firstly, NCI-H1299 cells were lysed with RIPA lysis buffer (Beyotime) including protease inhibitors. Afterward, cell lysate was incubated with A/G magnetic beads coated with anti-YAP. Finally, the magnetic beads were removed and the protein immunoprecipitation was obtained. Western blot was used to examine the levels of YAP, and TEAD was validated by western blot.

***In vivo* antitumor efficacy study.** 5–6 weeks male BALB/c nude mice (weight 18–20 g) were used for *in vivo* study. All mice obtained water and food at liberty under a 12 h light/12 h dark condition. A week later, the mice were fallen into four groups including control, oe-Sestrin2, oe-Sestrin2+oe-NC, oe-Sestrin2+oe-FOXM1. The nude mice received subcutaneous injection with NCI-H1299 cells (5.0×0^6/per mouse) with oe-Sestrin2, oe-Sestrin2+oe-NC, oe-Sestrin2+oe-FOXM1 transfection, respectively. Tumor volumes in nude mice were measured and recorded every 5 days. The tumor volume was calculated according to the formula of $V = length \times width^2/2$. After 30 d, the mice were euthanized and the tumors in the mice were collected. The tumor weight of tumors was measured. Notably, our study involving animal experiments was approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital of Hainan Medical University (No. HYLL-2022-207).

Statistical analysis. All data were presented as means ± standard deviation (SD). All experiments were repeated three times independently. GraphPad Prism 6 was used to analyze
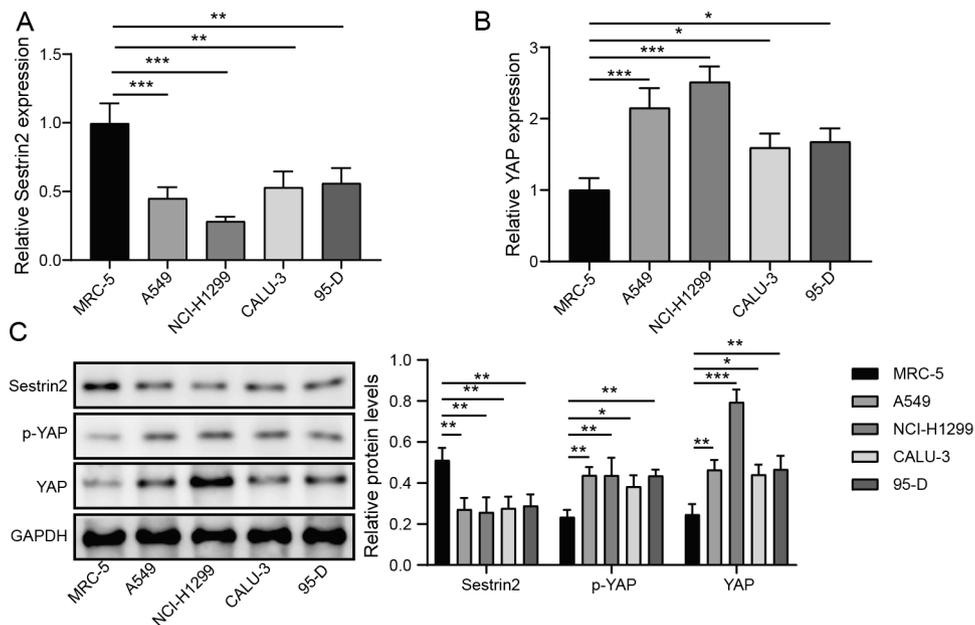

Figure 1. Sestrin2 expression was lowered while YAP expression was elevated in NSCLC cells. A, B) The mRNA levels of Sestrin2 and YAP were evaluated by qRT-PCR in cell lines of MRC-5, A549, NCI-H1299, CALU-3, 95-D. C) The protein levels of Sestrin2, YAP, and p-YAP were detected using western blot in cell lines of MRC-5, A549, NCI-H1299, CALU-3, and 95-D.

the data. A discrepancy between the two groups was analyzed by Student's t-test and more than two groups were analyzed by one-way analysis of variance (ANOVA). A p-value <0.05 was identified as a statistically significant difference.

Results

Sestrin2 expression was lowered while YAP expression was elevated in NSCLC cells. We detected the expression of Sestrin2 and YAP with qRT-PCR and western blot in NSCLC cell lines (A549, NCI-H1299, CALU-3, 95-D) and human embryonic lung fibroblasts cells (MRC-5). We observed Sestrin2 was evidently lessened while YAP and p-YAP were markedly elevated in NSCLC cell lines relative to MRC-5 cells (Figures 1A–1C). These results indicated Sestrin2, YAP might be closely implicated in the regulation of NSCLC. Since the significant differential expression of Sestrin2 and YAP was most obvious in NCI-H1299 cells compared to MRC-5 cells, NCI-H1299 cells were selected for subsequent experiments.

Sestrin2 overexpression resulted in the suppression of NSCLC cell proliferation, migration, invasion, and EMT. To uncover the specific role of Sestrin2 in NSCLC, the expression of Sestrin2 was overexpressed in NCI-H1299 cells. As depicted in Figures 2A and 2B, Sestrin2 was apparently enhanced by oe-Sestrin2 transfection. Besides, the influences of Sestrin2 overexpression on cell growth and metastasis were investigated. The results displayed that Sestrin2 sufficiency memorably impaired cell proliferation, migration, and invasion of NCI-H1299 cells (Figures 2C–2F). Furthermore, Sestrin2 overexpression inhibited the process of EMT. In detail, N-cadherin, Vimentin, Snail, and Twist were obviously repressed but E-cadherin was boosted by overexpressing Sestrin2 (Figure 2G). Yet, Sestrin2 overexpression has not influenced the mRNA expression of YAP (Figure 2H). Interestingly, the western blot exhibited that the protein level of p-AMPK was increased while the p-YAP protein was declined by Sestrin2 sufficiency (Figure 2I). Taken together, Sestrin2 overexpression could effectively suppress cell growth and metastasis of NCI-H1299 cells and might be correlated with regulating the AMPK/YAP pathway.

YAP knockdown restrained the capabilities of proliferation, migration, invasion, and EMT of NSCLC cells. Subsequently, we explored the role of YAP in NSCLC. Primarily, NCI-H1299 cells were transfected with sh-YAP to silence the expression of YAP. Compared with the sh-NC group, sh-YAP transfection distinctly lowered the expression of YAP and p-YAP (Figures 3A, 3B). Additionally, the abilities of cell proliferation, migration, and invasion were overtly repressed via the knockdown of YAP in NCI-H1299 cells (Figures 3C–3F). The EMT process was evidently suppressed with sh-YAP transfection by the decrease of N-cadherin, Vimentin, Snail, Twist, and the increase of E-cadherin (Figure 3G). In total, YAP knockdown restrained NSCLC cell growth and metastasis.

YAP mediated FOXM1 expression to promote cell proliferation, migration, invasion, and EMT of NSCLC cells via targeting TEAD. As previously depicted, YAP could combine with TEAD to mediate the expression of targeted genes [21]. We found that YAP could interact with TEAD according to the prediction of STRING, GeneMANIA, and hTFtarget (Figure 4A). Then, the Co-IP assay exhibited that the protein level of TEAD protein was pulled down by a specific YAP antibody, suggesting the interaction between YAP and TEAD (Figure 4B). We continued to excavate how TEAD affects YAP-mediated functions in NSCLC cells. Hence, NCI-H1299 cells were transfected with sh-YAP alone or together with oe-TEAD. sh-YAP transfection effectively reduced the mRNA and protein levels of YAP but didn't affect the mRNA and protein levels of TEAD. Likewise, oe-TEAD transfection merely sharply elevated the mRNA and protein levels of TEAD but failed to change the YAP expression (Figures 4C, 4D). Notably, FOXM1 expression was distinctly reduced by the YAP knockdown, whereas this phenomenon was compromised by overexpressing TEAD (Figures 4C, 4D). In addition, the protein expression of p-YAP was altered similarly to the changes of YAP (Figure 4D). Furthermore, TEAD sufficiency largely attenuated the suppressive impacts of YAP knockdown on cell migration and invasion (Figures 4E, 4F). Simultaneously, sh-YAP-mediated changes of EMT-associated proteins were abolished by TEAD overexpression (Figure 4G). To sum up, YAP elevated FOXM1 expression to promote NSCLC cell growth and metastasis by interacting with TEAD.

Sestrin2 repressed FOXM1 to suppress NSCLC cell proliferation, migration, invasion, and EMT by regulating the AMPK/YAP pathway. Aiming to figure out if FOXM1 is engaged in Sestrin2-mediated biological functions in NSCLC cells, NCI-H1299 cells were transfected with oe-Sestrin2 or co-transfected with oe-Sestrin2 and oe-FOXM1. Sestrin2 overexpression lowered the mRNA and protein levels of FOXM1 and the protein expression ratio of p-YAP/YAP but elevated the mRNA and protein levels of Sestrin2 and the protein expression ratio of p-AMPK/AMPK, while oe-FOXM1 transfection merely restored its reduced expression caused by Sestrin2 overexpression but had no effect on other proteins expression (Figures 5A, 5B). It was worth mentioning that FOXM1 sufficiency partly reversed overexpressing Sestrin2-suppressed migration, invasion, and EMT, which were evaluated by scratch test, Transwell as well as western blot, respectively (Figures 5C–5E). In conclusion, Sestrin2 restrained cell migration and invasion by regulating the AMPK/YAP/FOXM1 axis in NSCLC cells.

Sestrin2 restrained tumor growth *in vivo* by mediating the AMPK/YAP/FOXM1 axis. Finally, we verified the regulatory mechanism of Sestrin2 on tumor growth and the alteration of crucial molecules *in vivo* experiments. Nude mice received a subcutaneous injection of Sestrin2-overexpressed NCI-H1299 cells or Sestrin2 and FOXM1-overexpressed NCI-H1299 cells. After 1 month, we weighed the tumor and

depicted the volume growth curve. The results displayed that Sestrin2 sufficiency remarkably diminished tumor weight and volume compared to the control, but FOXM1 overexpression aggrandized the tumor weight and volume relative to the Sestrin2 sufficiency group (Figures 6A, 6B). In tumor tissues, Sestrin2 sufficiency elevated the mRNA and protein levels of Sestrin2, p-AMPK, E-cadherin, and lowered the

mRNA and protein levels of FOXM1 and the protein levels of p-YAP, N-cadherin, Vimentin, Snail, and Twist, which phenomena were rescued by FOXM1 overexpression in addition to having no influences of the expression of Sestrin2, p-AMPK, and p-YAP (Figure 6C–6E). Collectively, Sestrin2 restrained FOXM1 expression by the AMPK/YAP pathway to suppress tumor growth in mice inoculated with NSCLC cells.
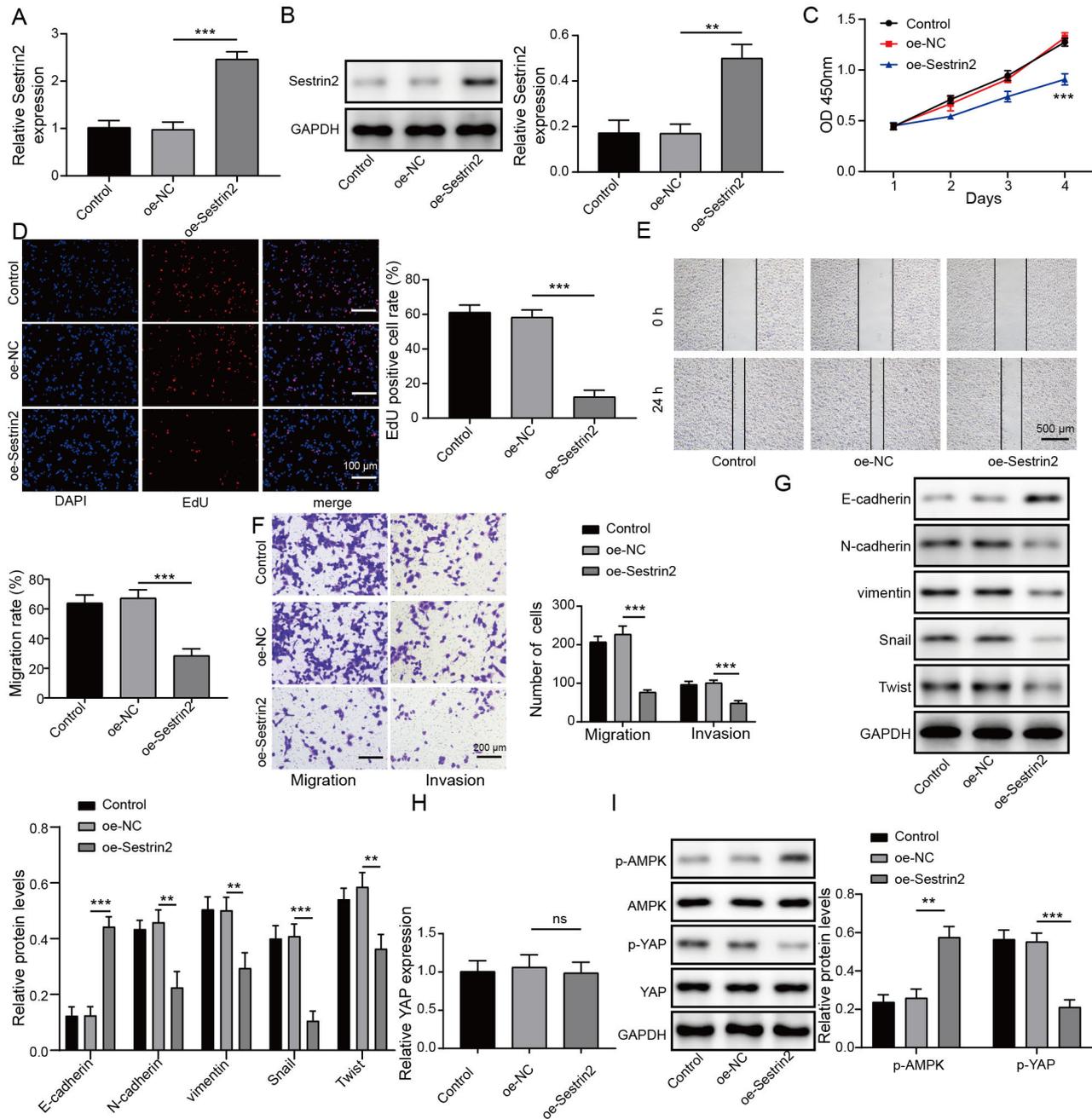

Figure 2. Sestrin2 overexpression resulted in the suppression of NSCLC cell proliferation, migration, invasion, and EMT. NCI-H1299 cells were transfected with oe-Sestrin2 plasmid. A, B) The transfection efficiency of overexpressing Sestrin2 was assessed by qRT-PCR and western blot. C, D) Cell proliferation was estimated using CCK-8 and EdU assay. E, F) The capabilities of migration and invasion were examined by scratch test and Transwell. G) The protein levels of E-cadherin, N-cadherin, Vimentin, Snail, and Twist were detected by western blot. H) The mRNA expression of YAP was detected using qRT-PCR. I) The protein levels of YAP, p-YAP, AMPK, and p-AMPK were detected using a western blot.

Discussion

NSCLC, as the main type of lung cancer, is a serious threat to the lives of patients, especially those with metastasis [22]. Accumulating evidence has determined that the EMT process prominently accelerates the metastasis of tumor cells and promotes tumor progression [23].

Suppressing the EMT process has been evidenced to be an effective approach for impairing NSCLC progression [24]. Therefore, in this study, we focused on the EMT process and attempted to seek out crucial molecules inhibiting the EMT process in NSCLC. Our findings revealed that Sestrin2 elevated FOXOM1 expression through mediating YAP-TEAD interaction to suppress NSCLC cell prolifera-
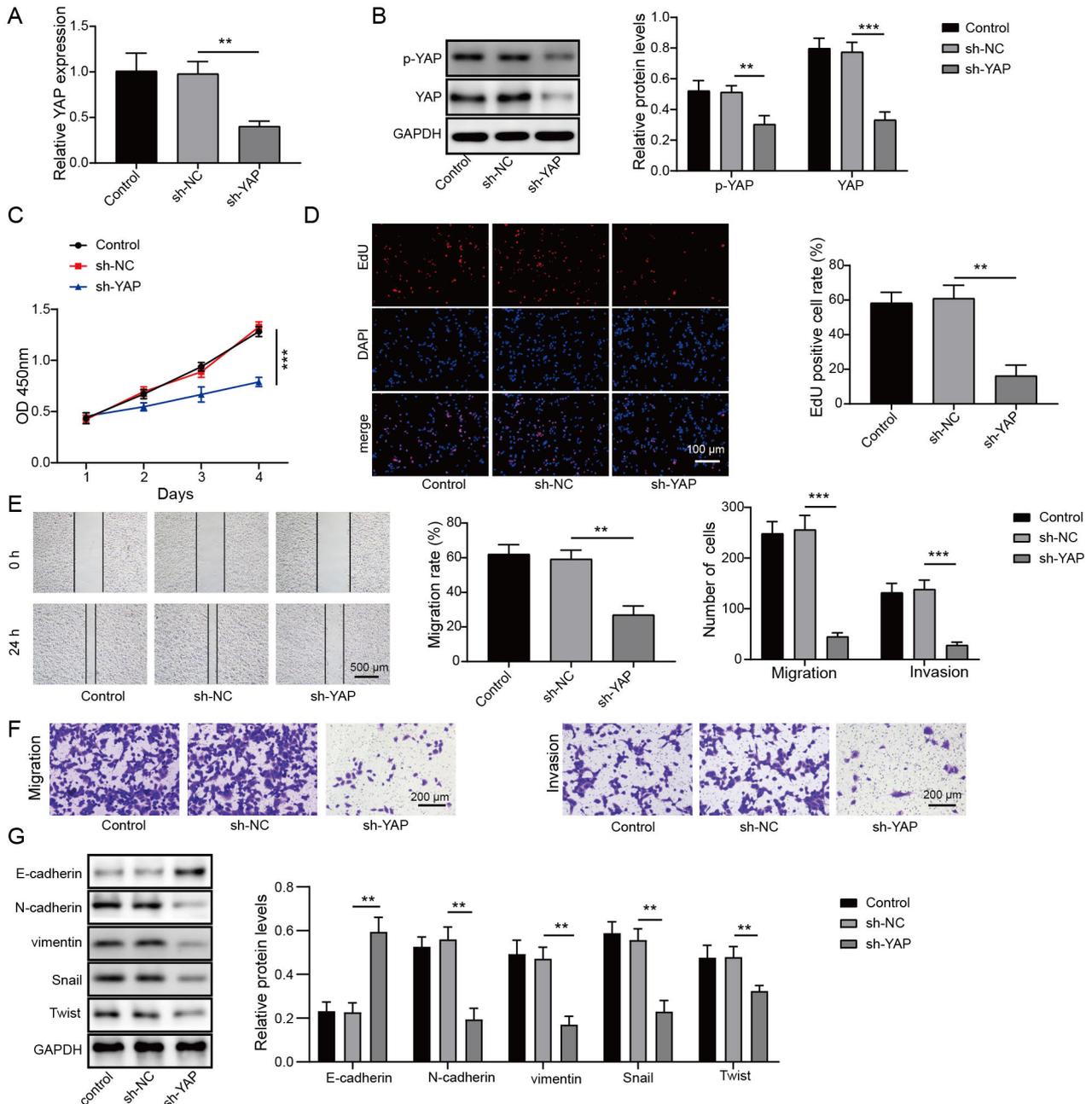

Figure 3. YAP knockdown restrained the capabilities of proliferation, migration, invasion, and EMT of NSCLC cells. NCI-H1299 cells were transfected with sh-YAP plasmid. A) The transfection efficiency of YAP knockdown was assessed by qRT-PCR. B) YAP and p-YAP expression were detected using a western blot. C, D) Cell proliferation was estimated using CCK-8 and EdU assay. E, F) The capabilities of migration and invasion were examined by scratch test and Transwell. G) The protein levels of E-cadherin, N-cadherin, Vimentin, Snail, and Twist were detected by a western blot.
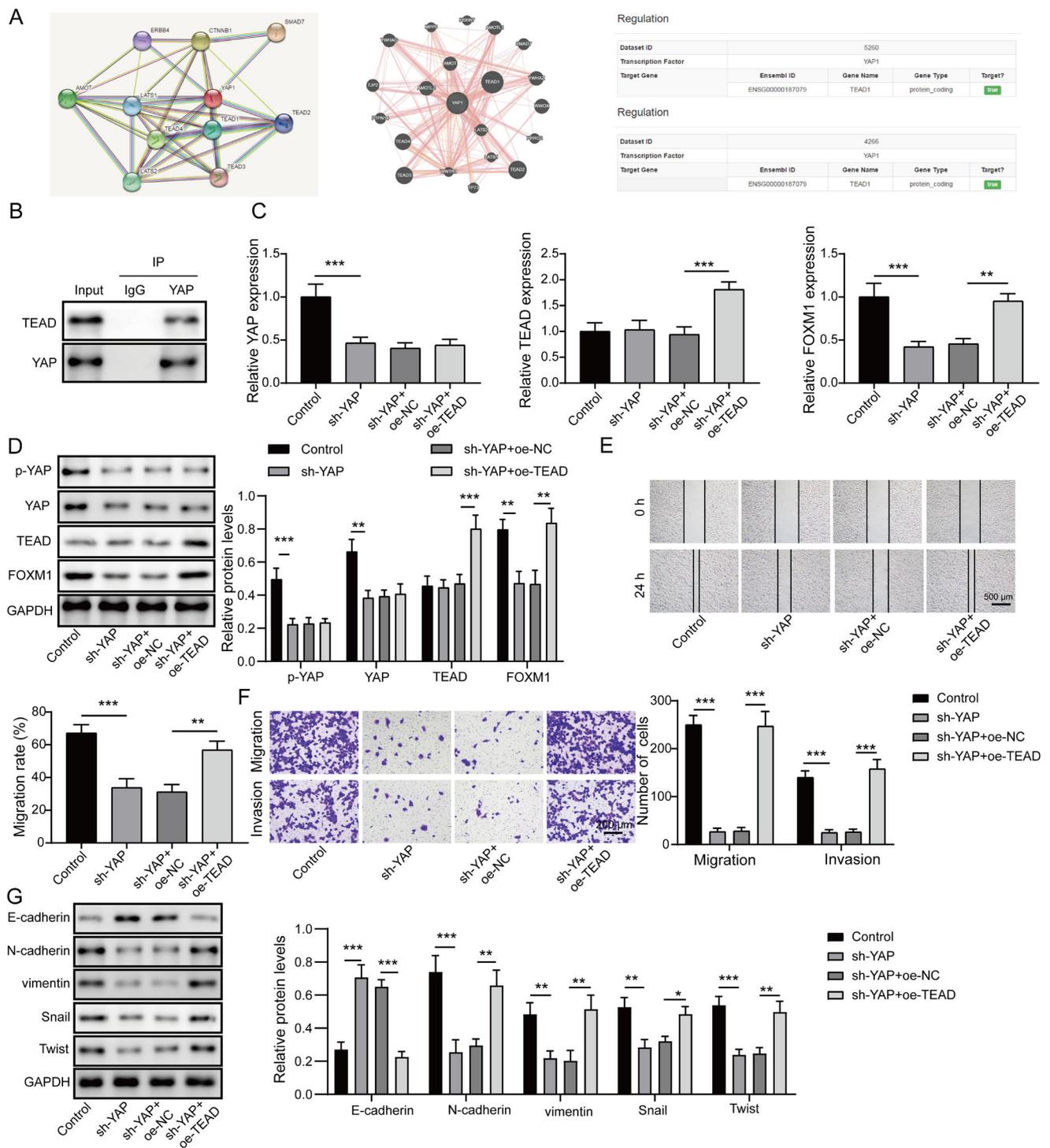
Figure 4. YAP mediated the FOXM1 expression to promote cell proliferation, migration, invasion, and EMT of NSCLC cells by targeting TEAD. A) The websites of STRING, GeneMANIA, and hTFtarget were performed to predict the combination between YAP and TEAD. B) Co-IP assay validated the combination between YAP and TEAD. NCI-H1299 cells were transfected with sh-YAP plasmid or together with oe-TEAD plasmid. C) The mRNA expression of YAP, TEAD, and FOXM1 was examined with qRT-PCR. D) The protein levels of p-YAP, YAP, TEAD, and FOXM1 were evaluated using a western blot. E, F) The capabilities of migration and invasion were examined by scratch test and Transwell. G) The protein levels of E-cadherin, N-cadherin, Vimentin, Snail, and Twist were detected by a western blot.
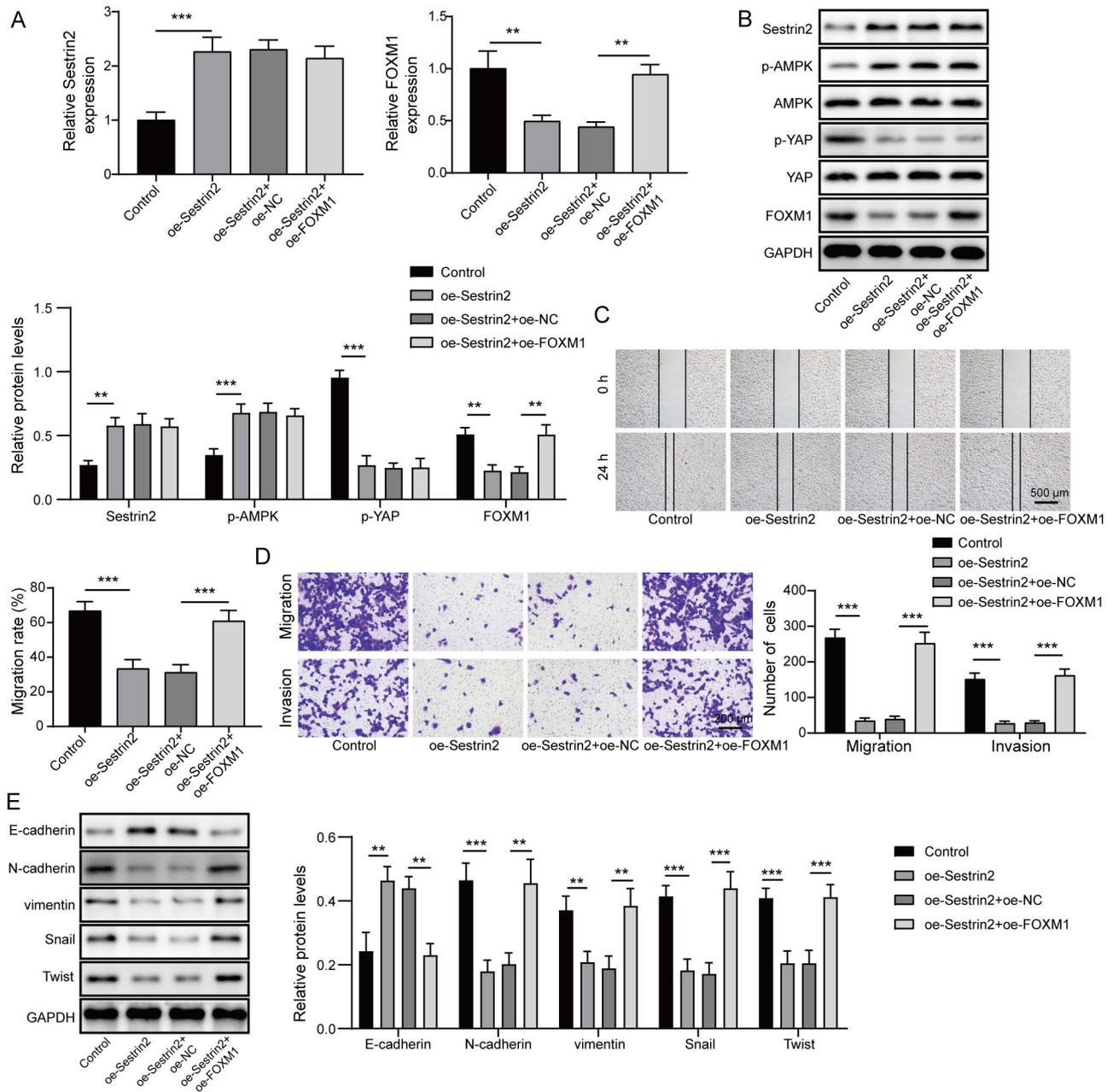
Figure 5. Sestrin2 repressed FOXM1 to suppress NSCLC cell proliferation, migration, invasion, and EMT by regulating the AMPK/YAP pathway. NCI-H1299 cells were transfected with oe-Sestrin2 plasmid or together with oe-FOXM1 plasmid. A) The mRNA expression of Sestrin2 and FOXM1 was examined with qRT-PCR. B) The protein levels of Sestrin2, YAP, p-YAP, AMPK, p-AMPK, and FOXM1 were evaluated using a western blot. C, D) The capabilities of migration and invasion were examined by scratch test and Transwell. E) The protein levels of E-cadherin, N-cadherin, Vimentin, Snail, and Twist were detected by a western blot.

tion, migration, invasion, and the EMT process, eventually restraining NSCLC progression.

Sestrin2, a conserved antioxidant and metabolic regulator, markedly reprogramed intracellular signaling pathways [25]. Currently, Sestrin2 was extensively explored in multi-diseases, such as diabetic cardiomyopathy, cholestatic liver injury, idiopathic pulmonary fibrosis, cancers

[5, 26–28]. A review proposed Sestrin2 could serve as a biomarker and therapeutic target in multiple diseases including cancers [5], which highlights the importance of Sestrin2 in the development of human diseases. Of note, Ala wrote a review published in 2022, and the content of this review mainly described and discussed the role of Sestrin2 in cancers as an oncogene and tumor suppressor gene [29].
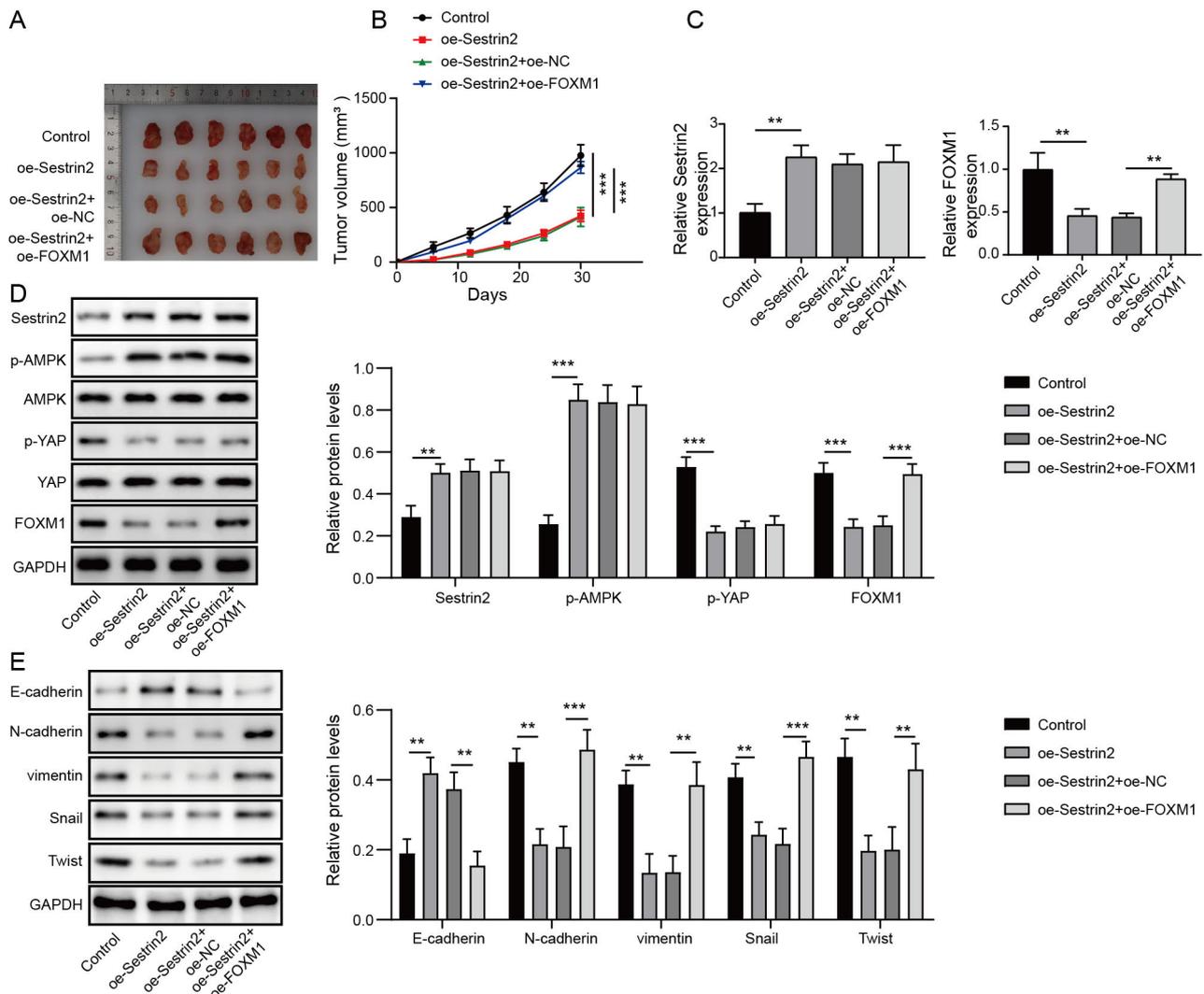
Figure 6. Sestrin2 restrained tumor growth *in vivo* by mediating the AMPK/YAP/FOXM1 axis. Nude mice were treated with subcutaneous injection with Sestrin2-overexpressed NCI-H1299 cells or both Sestrin2 and FOXM1-overexpressed NCI-H1299 cells. A) The tumor photographs. B) The growth curve of tumor volume. C) The mRNA expression of Sestrin2 and FOXM1 were examined with qRT-PCR. D) The protein levels of Sestrin2, YAP, p-YAP, AMPK, p-AMPK, and FOXM1 were evaluated using a western blot. E) The protein levels of E-cadherin, N-cadherin, Vimentin, Snail, and Twist were detected by a western blot.

Several studies have documented the roles of Sestrin2 and appeared opposite voices about the role of Sestrin2 in lung cancer. There were examples of the suppressive role of Sestrin2 in lung cancer. TP53 mutation/depletion led to a reduction of Sestrin2 to activate rapamycin complex 1 (mTORC1), thereby accelerating the progression of lung cancer [30]. Another study also revealed that Sestrin2 elevated cell apoptosis by affecting XIAP degradation in lung adenocarcinoma cells [31]. Chen et al. illustrated that in clinic samples from NSCLC patients, Sestrin2 had lower expression than that in non-cancerous tissues and closely correlated with favorable prognostic through evaluating the degree of tumor differentiation, tumor stage, and lymph node metastasis [8]. However, Chae et al. proposed that Sestrin2 expression was negatively correlated with the survival of lung cancer patients, and Sestrin2 knockdown could improve the chemotherapy resistance of cancer cells by reducing their stemness, proliferation, and migration of NSCLC cells [9]. So, does Sestrin2 promote or inhibit the progression of NSCLC? In our study, we found that Sestrin2 had lower expression in NSCLC cells compared to human embryonic lung fibroblasts cells. Moreover, Sestrin2 sufficiency could effectively repress cell proliferation, migration, invasion, and EMT processes in NSCLC cells, suggesting the inhibitory role of Sestrin2 in NSCLC. The pathological process and progression of NSCLC are influenced by various factors. Therefore, more studies are needed to explore the role of Sestrin2 in NSCLC from multi-aspects.

Currently, the downstream regulatory mechanisms of Sestrin2 have been reported in some studies. Sestrin2 inhibited cancer stemness by suppressing the Wnt/β-catenin signaling in colorectal cancer [7]. Sestrin2 promoted glutamine-dependent transcription of PGC-1α and elevated the survival of liver cancer cells under glucose limitation [32]. In lung adenocarcinoma, Sestrin2 could regulate XIAP degradation to promote cytokine-induced cell death [31]. However, there were few studies on the specific molecular regulatory mechanism of Sestrin2, which was worthy of more studies to be explored. YAP is a crucial molecule in the Hippo signaling pathway which engaged in the progression of cancers [33, 34]. As expected, the expression and functions of YAP in NSCLC have been reported. For example, Niu et al. expounded that YAP expression was elevated in NSCLC and regulated by the circ_0014235/miR-146b-5p axis; elevation of YAP could promote PD-L1 to raise gefitinib IC50 and strengthen malignant behaviors in NSCLC [35]; which indicated YAP was an oncogene existing in NSCLC. In this study, our result regarding YAP expression was consistent with the prior research [35]. The expression of YAP was greatly enhanced in NSCLC cells relative to human embryonic lung fibroblasts. Further experiments demonstrated YAP knockdown weakened the capabilities of proliferation, migration, invasion, and EMT in NSCLC cells. Amazingly, a previous study pointed out that Sestrin2 promoted the ability of corneal epithelial cell proliferation by suppressing YAP activation [16]. Furthermore, the activation of AMPK could inhibit YAP expression to hinder oncogenic transformation [36]. Thus, we conjectured Sestrin2 might affect YAP activity by mediating the phosphorylation of AMPK to promote cell growth and the EMT process of NSCLC. Here, Sestrin2 overexpression elevated AMPK phosphorylate and inhibited YAP phosphorylate, indicating Sestrin2 indirectly and negatively influenced YAP expression through regulation of the activation of AMPK.

FOXM1 is a member of Forkhead box transcription factors, which are widely engaged in various pathological processes such as EMT [37]. Massive evidence has demonstrated FOXM1 was a cancerogenic element in multifarious tumors including colon cancer, NSCLC [38, 39]. For instance, inhibiting FOXM1 expression could suppress cell proliferation and reinforce the pesticide effect of 5-FU on colon cancer [38]. Additionally, FOXM1 as a downstream gene of circRNA ZNF609/miR-623 destroyed the suppressive influences of circRNA ZNF609 silencing on NSCLC cell growth [39]. In the current work, our findings revealed that FOXM1 sufficiency reversed Sestrin2 overexpression-mediated inhibitory impacts on NSCLC cell growth, metastasis, and EMT process *in vitro*. Meanwhile, FOXM1 could abolish Sestrin2 upregulation-mediated suppression of solid tumor volume of mice with NSCLC cell inoculation. Subsequently, we were curious about which regulated the expression of FOXM1 in NSCLC. According to previous studies, for example, in pulmonary arterial hypertension, Galectin-3 triggered YAP activation

to elevate FOXM1 expression [40]. Another study figured out that FOXM1 was identified as YAP-dependent crucial molecule in epidermal stem cells [41]. It was reported that the expression of numerous genes was regulated by YAP-TEAD transcriptional complex and FOXM1 was a gene regulated by the YAP-TEAD complex in pancreatic ductal adenocarcinoma [42]. In our study, the interaction between YAP and TEAD was verified by the Co-IP assay. YAP-TEAD complex positively mediated FOXM1 expression in NSCLC. Moreover, TEAD upregulation rescued the repressive influences of YAP knockdown on NSCLC cell growth and metastasis.

In conclusion, we were the first to propose that Sestrin2 activated AMPK phosphorylate to impair YAP activation and then suppressed cell proliferation, migration, invasion, and EMT processes of NSCLC cells by regulating the YAP-TEAD/FOXM1 axis. Our discoveries illustrated the mechanism of Sestrin2 and provided a novel therapeutic target for NSCLC treatment.

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