


RESEARCH

Open Access



Circular RNA circ-CPA4/ let-7 miRNA/PD-L1 axis regulates cell growth, stemness, drug resistance and immune evasion in non-small cell lung cancer (NSCLC)

Weijun Hong^{1†}, Min Xue^{1†}, Jun Jiang², Yajuan Zhang^{1*} and Xiwen Gao^{1*} 

Abstract

Background: Non-small cell lung cancer (NSCLC) cells derived intracellular and extracellular programmed cell death ligand 1 (PD-L1) promoted cancer progression and drug resistance, and facilitated tumor immune evasion. However, the detailed molecular mechanisms are still largely unknown. In the present study, we aimed to explore the role of circular RNA circ-CPA4/let-7 miRNA/PD-L1 axis in the regulation of NSCLC progression, drug resistance and tumor immune microenvironment.

Methods: Real-Time qPCR and Western Blot analysis were conducted to examine gene expressions at transcriptional and translated levels, respectively. The regulatory mechanisms of circ-CPA4, let-7 miRNA and PD-L1 were validated by dual-luciferase reporter gene system and RNA pull-down assay. Cell growth and apoptosis were determined by CCK-8 assay, colony formation assay and Annexin V-FITC/PI double staining assay. Cell mobility was evaluated by transwell assay.

Results: Circ-CPA4 and PD-L1 were high-expressed, while let-7 miRNA was low-expressed in NSCLC cells and cancer tissues compared to the human bronchial epithelial (HBE) cells and their paired clinical normal adjacent tissues, respectively. Besides, knock-down of circ-CPA4 inhibited cell growth, mobility and epithelial-mesenchymal transition (EMT), and promoted cell death in NSCLC cells by downregulating PD-L1 through serving as a RNA sponge for let-7 miRNA. In addition, the NSCLC cells derived PD-L1-containing exosomes promoted cell stemness and increased resistance of NSCLC cells to cisplatin. Notably, by co-culturing the NSCLC cells with CD8⁺ T cells isolated from human peripheral blood mononuclear cells (hPBMCs) in a transwell co-culturing system, we found that NSCLC cells inactivated CD8⁺ T cells in a secreted PD-L1-dependent manner. Further results suggested that circ-CPA4 also positively regulated exosomal PD-L1, and the NSCLC cells with circ-CPA4 ablation re-activated CD8⁺ T cells in the co-culturing system.

Conclusion: Taken together, circ-CPA4 regulated cell growth, mobility, stemness and drug resistance in NSCLC cells and inactivated CD8⁺ T cells in the tumor immune microenvironment through let-7 miRNA/PD-L1 axis.

Keywords: NSCLC, Immune evasion, Stemness, PD-L1, Circ-CPA4, Let-7 miRNA

* Correspondence: zhang_yajuan221@126.com; gaoxiwen@sina.com

[†]Weijun Hong and Min Xue are co-first authors.

¹Department of Respiratory Medicine, Minhang Hospital, Fudan University, 170 Xin-Song Road, Shanghai 201199, China

Full list of author information is available at the end of the article



© The Author(s). 2020 **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

Background

Tumor immune microenvironment was crucial for the development of non-small cell lung cancer (NSCLC) [1, 2], and NSCLC cells interacted with immune cells to facilitate immune evasion [3]. Based on this, current cancer immunotherapies were developed to treat NSCLC in clinical practices [4, 5]. Among those drugs and therapies, programmed death-1 (PD-1)/programmed death ligand-1 (PD-L1) blockade was proved to be novel cancer immunotherapies for cancers treatment, and the associated drugs were developed to treat NSCLC in clinic [6, 7]. These therapeutic drugs included PD-1 inhibitors Nivolumab (Opdivo) [8, 9] as well as Pembrolizumab (Keytruda) [10, 11], and PD-L1 inhibitors Atezolizumab (Tecentriq) [12], Durvalumab (Imfinzi) [13] and Avelumab (Bavencio) [14]. Previous data indicated that cancer cells derived PD-L1 regulated the activity of CD8⁺ T cell activity [15, 16], which was a pivotal component of the cellular immune response in tumor development [17, 18]. Mechanistically, cancer cells derived PD-L1 induced dysfunctions of CD8⁺ T cell contributed to immune evasion and disabled immune surveillance [19]. Aside from regulating immune evasion of cancer cells, PD-L1 also directly regulated cancer cell functions and drug resistance [20, 21]. Previous data indicated that upregulation of PD-L1 promoted cancer cell growth and motility [22], and sustained stemness of breast cancer cells to facilitate drug resistance [20, 21, 23].

MicroRNAs (miRNAs) had widely been reported to regulate NSCLC progression [24]. For example, overexpression of miR-433 inhibited the development of NSCLC by targeting Smad2 [24] and miR-421 played an oncogenic role in NSCLC progression [25]. Among all the miRNAs, let-7 miRNA served as a tumor suppressor in multiple cancers [26–28], such as bladder cancer [27], ovarian cancer [26] and NSCLC [28]. Specifically, upregulated let-7 inhibited migration and invasion of NSCLC cells by synergistically targeting ITGB3 and MAP4K3 [28]. Notably, previous study proved that the 3'UTR region of PD-L1 mRNA could be sponged by let-7 miRNA, and the expression levels of PD-L1 could be inhibited by overexpressing let-7 miRNA [29]. In addition, let-7 miRNA participated in the regulation of cancer immunotherapy [30] and modulated the biological functions of T cells in colorectal cancer microenvironment [31].

Aberrant expressions of circular RNAs (circRNAs) were also related with the development of cancers, and recent studies focused on investigating the role of circRNAs in regulating NSCLC pathogenesis [32, 33]. For instance, the circRNA circP4HB promoted NSCLC aggressiveness and metastasis [32] and circ-FOXM1 facilitated NSCLC cell proliferation [33]. Circular RNA circ-CPA4 (hsa_circ_0082374) had recently been

identified as an oncogene in glioma [34], however, little known about the role of this circRNA in regulating progression of other types of cancer. Besides, circRNAs were known as “sponges” for miRNAs, and circRNAs regulated the levels of their downstream miRNAs in a competing endogenous RNA (ceRNA) dependent manner [35–37]. In particular, let-7 miRNA could be sponged and inhibited by circ-CPA4 in the development of glioma [34]. Therefore, it is reasonable to speculate that circ-CPA4 might involve in the modulation of NSCLC progression by sponging let-7 miRNA, however, detailed mechanisms are still largely unknown.

Based on the existed information, this study validated that circular RNA circ-CPA4 regulated NSCLC progression, drug resistance and the biological functions of tumor associated immune cells through targeting let-7 miRNA/PD-L1 axis. Our work broadened our knowledge of NSCLC pathogenesis, and provided potential therapeutic agents for this disease.

Materials and methods

Clinical specimens collection

The clinical cancer tissues and their paired adjacent normal tissues were collected from NSCLC patients ($N=50$) in Minhang Hospital, Fudan University, from 2013 to 2016. The clinicopathological features of the patients were listed in Table 1, Table 2 and Table 3. All the clinical experiments in this study were approved by the Ethics Committee of Minhang Hospital, Fudan University (No. 2018FU00732974). Besides, the written informed consent was obtained from all the participants involved in this study.

Cell culture and vectors transfection

The human NSCLC cell lines (A549, H1299, SK-MES-1 and Calu-3) and human normal bronchial epithelial cell line (HBE) were purchased from American Type Culture Collection (ATCC, USA). All the cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) culture medium under the standard conditions with 5% CO₂ atmosphere with 37 °C. The vectors for overexpression and downregulation circ-CPA4 and PD-L1 were designed and constructed by Sangon Biotech (Shanghai, China). In addition, the let-7 miRNA mimic and inhibitor were obtained from Ribobio (Guangzhou, China). The detailed information of the above vectors were listed in Table 4. The above vectors were delivered into the NSCLC cells by using the Lipofectamine reagent purchased from Invitrogen (CA, USA).

Real-time qPCR

The total RNA were extracted from cells and tissues by using the Trizol kit obtained from Invitrogen (USA) according to the manufacturer's protocol. The Real-Time

Table 1 The clinicopathological features of NSCLC patients with differential expressions of circular RNA circ-CPA4

Parameters	Cases	circular RNA circ-CPA4		P value
		High	Low	
Age				0.721
≥ 55	35	20	15	
< 55	15	5	10	
Gender				
Male	25	13	12	0.832
Female	25	12	13	
TNM stage				0.023
I/II	18	9	9	
III/IV	32	16	16	
Pathological Type				0.593
Squamous	21	10	11	
Adenocarcinoma	12	7	5	
Large Cell Lung cancer	17	8	9	
Smoking status				0.098
Non-smoker	14	7	7	
Smoker	16	7	9	
Heavy smoker	20	11	9	
Lymphatic Metastasis				0.019
No	29	13	16	
Yes	21	12	9	

Table 2 The clinicopathological features of NSCLC patients with differential expressions of let-7 miRNA

Parameters	Cases	Let-7 miRNA		P value
		High	Low	
Age				0.093
≥ 55	35	18	17	
< 55	15	8	7	
Gender				0.482
Male	25	13	12	
Female	25	13	12	
TNM stage				0.005
I/II	18	14	4	
III/IV	32	12	20	
Pathological Type				0.983
Squamous	21	12	9	
Adenocarcinoma	12	5	7	
Large Cell Lung cancer	17	9	8	
Smoking status				0.447
Non-smoker	14	7	7	
Smoker	16	6	10	
Heavy smoker	20	13	7	
Lymphatic Metastasis				0.042
No	29	14	15	
Yes	21	12	9	

qPCR was conducted to examine the expression levels of circ-CPA4 and let-7 miRNA, and mRNA levels for PD-L1, OCT4, SOX2, Nanog, ALDH1, IFN- γ , IL-4 and IL-10 according to the procedures provided by the previous study [38]. For circ-CPA4 quantification, the circular RNA were enriched and pretreated with RNase R enzyme (3 U/ μ g) for 20 min at 37 °C to eliminate linear CPA4. The primer sequences for the above genes were listed in Table 5.

Western blot

The total proteins of the cells and clinical tissues were extracted by using the RIPA lysis buffer solution (Beyotime, China) according to the manufacturer's protocol. Based on the protocols provided by the previous study [38], Western Blot was used to determine the expression levels of proteins involved in this study. The primary antibodies against PD-L1 (1:1000, Abcam, UK), β -actin (1:2000, Abcam, UK), Cyclin D1 (1:1500, Abcam, UK), Bax (1:1000, Abcam, UK), Bcl-2 (1:2000, Abcam, UK), N-cadherin (1:1500, Abcam, UK), Vimentin (1:1000, Abcam, UK) and TSG101 (1:1500, Abcam, UK) were purchased. The horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000, Abcam,

UK) was also obtained. Finally, the protein bands were visualized by using a electrochemiluminescence (ECL) system and the grey values were measured by Image J software to evaluate relative protein levels, and normalized to β -actin.

Dual-luciferase reporter gene system

The online starBase software (<http://starbase.sysu.edu.cn/>) was used to predict the binding sites of let-7 miRNA with wild type circ-CPA4 (Wt-circ-CPA4) and 3' untranslated regions of PD-L1 mRNA (Wt-PD-L1), respectively. The targeting sites were mutated in circ-CPA4 (Mut-circ-CPA4) and PD-L1 mRNA (Mut-PD-L1), and the above sequences were cloned into a PmiR-RB-REPORT™ plasmid (RiboBio, Guangdong, China) to generate reporter vectors. The above vectors were co-transfected with let-7 miRNA mimic and inhibitor into NSCLC cells, respectively. A luciferase detection kit (Beyotime, Shanghai, China) was used to detect the relative luciferase activity in cells.

Pull-down assay

The biotin-labeled probes for circ-CPA4 and 3' UTR region of PD-L1 were designed and synthesized by Sangon

Table 3 The clinicopathological features of NSCLC patients with differential expressions of PD-L1 mRNA

Parameters	Cases	PD-L1 mRNA		P value
		High	Low	
Age				0.062
≥ 55	35	16	19	
< 55	15	5	10	
Gender				0.523
Male	25	14	11	
Female	25	7	18	
TNM stage				0.031
I/II	18	12	6	
III/IV	32	9	23	
Pathological Type				0.511
Squamous	21	8	13	
Adenocarcinoma	12	7	5	
Large Cell Lung cancer	17	6	11	
Smoking status				0.345
Non-smoker	14	6	8	
Smoker	16	7	9	
Heavy smoker	20	8	12	
Lymphatic Metastasis				0.011
No	29	7	22	
Yes	21	14	7	

Biotech (Shanghai, China). The pull-down assay was conducted to assess their binding abilities with let-7 miRNA. Briefly, the cells were fixed, lysed and centrifuged, the supernatants were used as input, and the above probes were incubated with the supernatants overnight at room temperature. After that, the lysis buffer and Proteinase K were used to reverse the formaldehyde crosslinking. Real-Time qPCR was conducted to examine let-7 miRNA levels.

Isolation and purification of exosomes

The exosomes in the supernatants were isolated and purified according to the previous study [39]. Briefly, culture supernatants were centrifuged at 2000 g for 20 min, and micro-vesicles were pelleted after

Table 4 Sequences of siRNAs, let-7 miRNA mimic and inhibitor

Gene	Primer sequences (strand)
PD-L1	Forward: 5'-GGUAAGAACAUAUUAUCAAdTdT-3' Reverse: 5'-UUGAAUAUUGUUCUUAUCCdTdT-3'
Si-circ-CPA4	5'-UUCUCCGAACGUGUCACGUTT-3'
Mimic	5'-UGAGGUAGUAGGUUGUAUGGUU-3'
Inhibitor	5'-AACCAUCAACCUACUACCUCA-3'

Table 5 Primer sequences for Real-Time qPCR

Gene	Primer sequences (strand)
β-actin	Forward: 5'-CTCCATCCTGGCCTCGCTGT-3' Reverse: 5'-GCTGCTACCTTCACCGTTCC-3'
U6	Forward: 5'-GACTATCATATGCTTACCGT-3' Reverse: 5'-GGCAGGAAGAGGGCCTAT-3'
Circ-CPA4	Forward: 5'-ACAGCATCTGGTGTGTGCTT-3' Reverse: 5'-CCCTTCTCGCAAACTAGC-3'
Let-7	Forward: 5'-AGCAAGCTTTGGCACCCACCGTAGAAC-3' Reverse: 5'-TAAGGATCCGATGCAGGGACAAGGACAGAA-3'
PD-L1	Forward: 5'-GCCGAAGTCATCTGGACAAG-3' Reverse: 5'-TCTCAGTGTCTGGTCACAT-3'
OCT4	Forward: 5'-AGCGATCAAGCAGCGACTA-3' Reverse: 5'-GGAAAGGGACCGAGGAGTA-3'
SOX2	Forward: 5'-CATCACCCACAGCAAATGAC-3' Reverse: 5'-CAAAGTCTCTACCGTACCCTACT-3'
Nanog	Forward: 5'-GCAGGCAACTCACTTTATCC -3' Reverse: 5'-CCCACAATACAGGCATAG-3'
ALDH1	Forward: 5'-AGCCTTACAGGATCAACAGA-3' Reverse: 5'-GTCGGCATCAGCTAACACAA-3'
IFN-γ	Forward: 5'-CCGAAACAGGCTCCCAACCG-3' Reverse: 5'-GGGAAGACGCTCTCACCT-3'
IL-4	Forward: 5'-AACGGCTCGACAGGAACCT-3' Reverse: 5'-ACTCTGGTTGGCTTCTCTCCA-3'
IL-10	Forward: 5'-ACAGCCGGGAAGACAATAAC-3' Reverse: 5'-CAGCTGGTCTTTGTTTAAA-3'

centrifugation at 16,500 g for 45 min and re-suspended in phosphate buffer saline (PBS). After that, the supernatants were centrifuged at 100,000 g for 2 h at 4 °C and the exosomes were suspended in PBS and collected by ultracentrifugation at 100,000 g for 2 h. Finally, the exosomes were purified by using the commercial exosome isolation kit (Invitrogen, USA) in keeping with the manufacturer's protocol.

Cell counting kit-8 (CCK-8) assay

The cells were collected and cell proliferation abilities were measured by using the commercial CCK-8 kit (YEASEN, Shanghai, China) based on the protocol provided by the manufacturer. Briefly, the cells were incubated with the CCK-8 reaction solution for 2 h at 37 °C. After that, the optical density (OD) values were measured in the wavelength of 450 nm to quantify the proliferation abilities of cells.

Trypan blue assay

Cell viability was determined by using the trypan blue staining assay. Briefly, the cells were collected and

stained with trypan blue solution (LEAGENE, Beijing, China) according to the manufacturer's instruction. The cells were observed under the light microscope and the dead blue cells were counted. Cell viability was calculated by using the following formula. Cell viability (%) = (Total cells – dead cells)/Total cells × 100%.

Flow cytometry (FCM) assay for cell apoptosis

The Annexin V-FITC/Propidium iodide (PI) double staining kit (YEASEN, Shanghai, China) was employed to examine cell apoptosis in keeping with the protocol provided by the manufacturer. Briefly, the cells were harvested and stained with Annexin V-FITC and PI respectively, the FCM (Partec, Germany) was employed to measure cell apoptosis ratio. The FITC was detected at 530 nm, and PI was detected at 575 nm, respectively.

Colony formation assay

The NSCLC cells (A549 and H1299) were seeded into the 6-well plates at the density of 1000 cells per well. Subsequently, the plates were placed in the incubator with standard culture conditions for 14 days, and stained with 0.1% crystal violet (Beyotime, Shanghai, China) for 1 h. Finally, the colonies containing at least 15 cells were counted by a gel documentation system purchased from BioRad (USA).

Transwell assay

The transwell assay was performed to evaluate cell invasion and migration abilities according to the previous study [40]. Briefly, the NSCLC cells were placed in the upper chamber with serum-free RPMI 1640 and the lower chamber was filled with culture medium with 15% fetal bovine serum (FBS). The cells in the Matrigel (Sigma-Aldrich, USA) were fixed by 4% paraformaldehyde and stained with 0.1% crystal violet (Beyotime, Shanghai, China). The cells were observed and photographed under light microscope, and 10 fields were selected to count the cells to reflect cell mobility.

Enzyme linked immunosorbent assay (ELISA)

The expression levels of inflammatory cytokines (IFN- γ , IL-4 and IL-10) in the supernatants of the co-culturing system were measured by using the commercial ELISA kits purchased from RAPIDBIO (CA, USA) based on the protocols provided by the manufacturer. Briefly, the supernatants were collected and incubated with reaction solution and subsequently with stop solution, the optical density (OD) values were measured by using a microplate reader (Molecular Devices, CA, USA) at 450 nm to evaluate the expression levels of above cytokines.

Isolation and culture of CD8⁺ T cells from peripheral blood

The blood was collected from normal volunteers, and human peripheral blood mononuclear cells (hPBMCs) were isolated and purified by using the Ficoll kit (Sigma-Aldrich, USA) according to the manufacturer's instruction. After that, the CD8⁺ T cells were isolated from the hPBMCs by using a flow cytometer (Partec, Germany) based on the protocol provided by the previous study [41].

Establishment of xenograft mice models

The nude mice (age 6–8 weeks) were purchased from the Experimental Animal Center of Fudan University and all the animal experiments were approved by the Animal Management Center of Minhang Hospital, Fudan University. The NSCLC cells (A549 and H1299) were transfected with vectors, and subcutaneously injected into the back flanks of the mice for 14 days. After that, the mice were sacrificed, the tumor weight was measured and the tissues were collected for Western Blot analysis. All the animal experiments were in accordance with the ethical manual for laboratory animals.

Data collection and analysis

The data of the experiment results were collected and represented as Means \pm Standard Deviation (SD), and the data were analyzed by using the SPSS 18.0 software. The Student's t-test was used to compare the differences between two groups, and the one-way ANOVA analysis was conducted to compare multiple groups. In addition, Pearson correlation analysis was conducted to determine the correlations among circ-CPA4, let-7 miRNA and PD-L1 mRNA in NSCLC clinical samples, and Kaplan-Meier survival analysis was conducted to investigate the association of the above 3 genes with patients prognosis. In addition, the Pan-cancer analysis (<http://starbase.sysu.edu.cn/panCancer.php>) was conducted to analyze the correlations of circ-CPA4, let-7 miRNA and PD-L1 (CD274) with lung squamous cell carcinoma (LUSC) progression. Each experiment in this study repeated at least 3 times. * $P < 0.05$, ** $P < 0.01$ and "NS" means "no statistical significance".

Results

The expression levels of circ-CPA4, let-7 miRNA and PD-L1 in NSCLC tissues and cell lines

The NSCLC tissues ($N = 50$) and their paired adjacent normal tissues ($N = 50$) were collected, the expression levels of circ-CPA4 (Fig. 1a), let-7 miRNA (Fig. 1b) and PD-L1 mRNA (Fig. 1c) were determined, respectively. The results indicated that circ-CPA4 and PD-L1 mRNA were high-expressed, while let-7 miRNA was low-expressed in the NSCLC tissues compared to the their

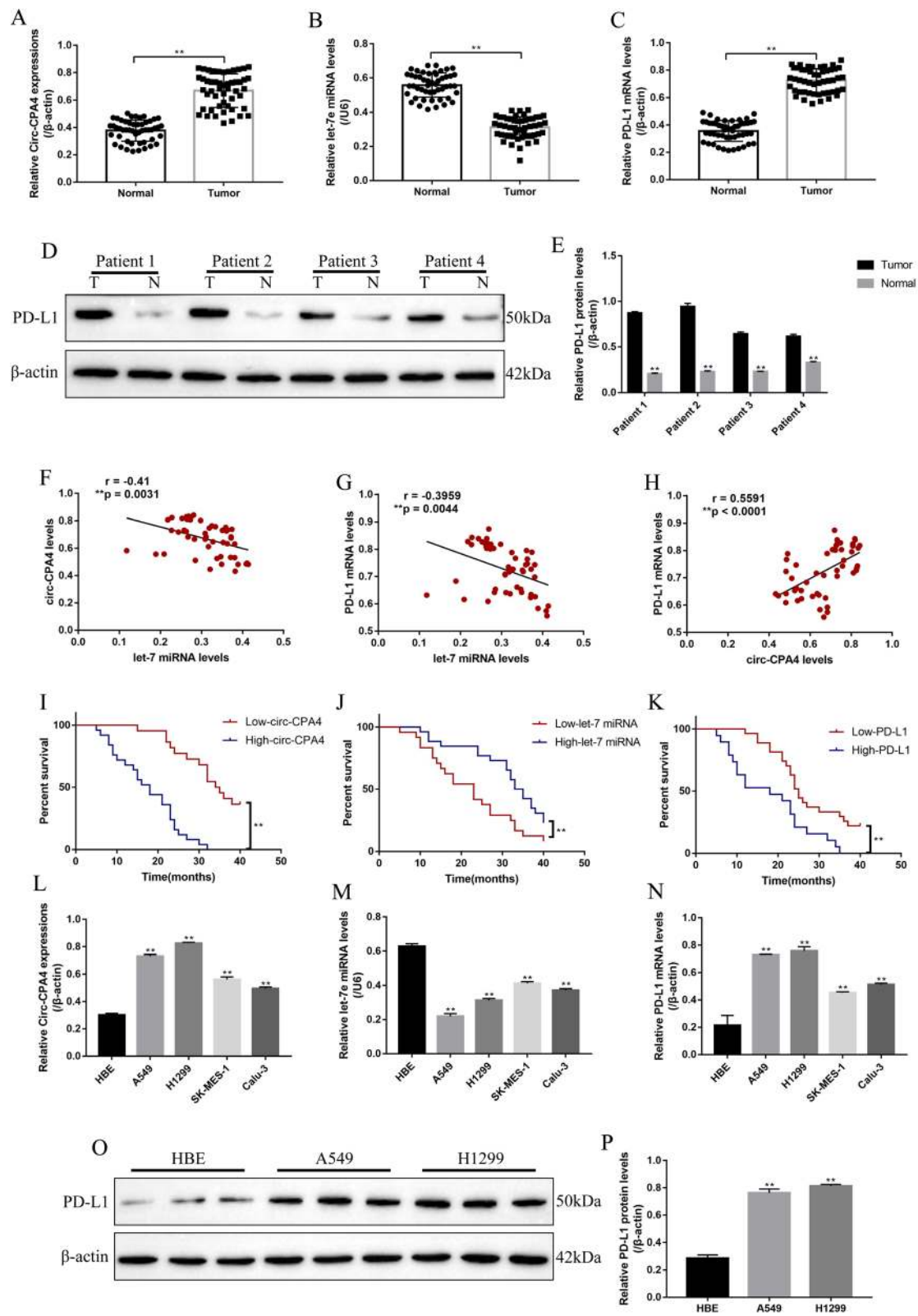


Fig. 1 (See legend on next page.)

(See figure on previous page.)

Fig. 1 The expression status and correlation of circ-CPA4, let-7 miRNA and PD-L1 in NSCLC tissues and cell lines. The NSCLC tissues ($N = 50$) and their paired adjacent normal tissues ($N = 50$) were collected, and Real-Time qPCR was conducted to determine the expression levels of **a** circ-CPA4, **b** let-7 miRNA and **c** PD-L1 mRNA in NSCLC tissues and their paired adjacent normal tissues. **d, e** The clinical samples collected from four patients, and the expression levels of PD-L1 protein were measured by using Western Blot. ("T" represented "tumor tissues", "N" represented "normal tissues"). Pearson correlation analysis was conducted to analyze the correlations of **f** circ-CPA4 and let-7 miRNA, **g** let-7 miRNA and PD-L1 mRNA and **h** circ-CPA4 and PD-L1 mRNA in NSCLC tissues. Kaplan-Meier survival analysis was performed to analyze the correlations of **i** circ-CPA4, **j** let-7 miRNA and **k** PD-L1 with NSCLC patients prognosis. Real-Time qPCR was used to measure the expressions of **l** circ-CPA4, **m** let-7 miRNA and **n** PD-L1 mRNA in the human NSCLC cell lines (A549, H1299, SK-MES-1 and Calu-3) and human normal bronchial epithelial cell line (HBE). **o, p** Western Blot was conducted to examine PD-L1 protein expressions in HBE, A549 and H1299 cells. Each experiment repeated at least 3 times. * $P < 0.05$, ** $P < 0.01$, NS means no statistical significance

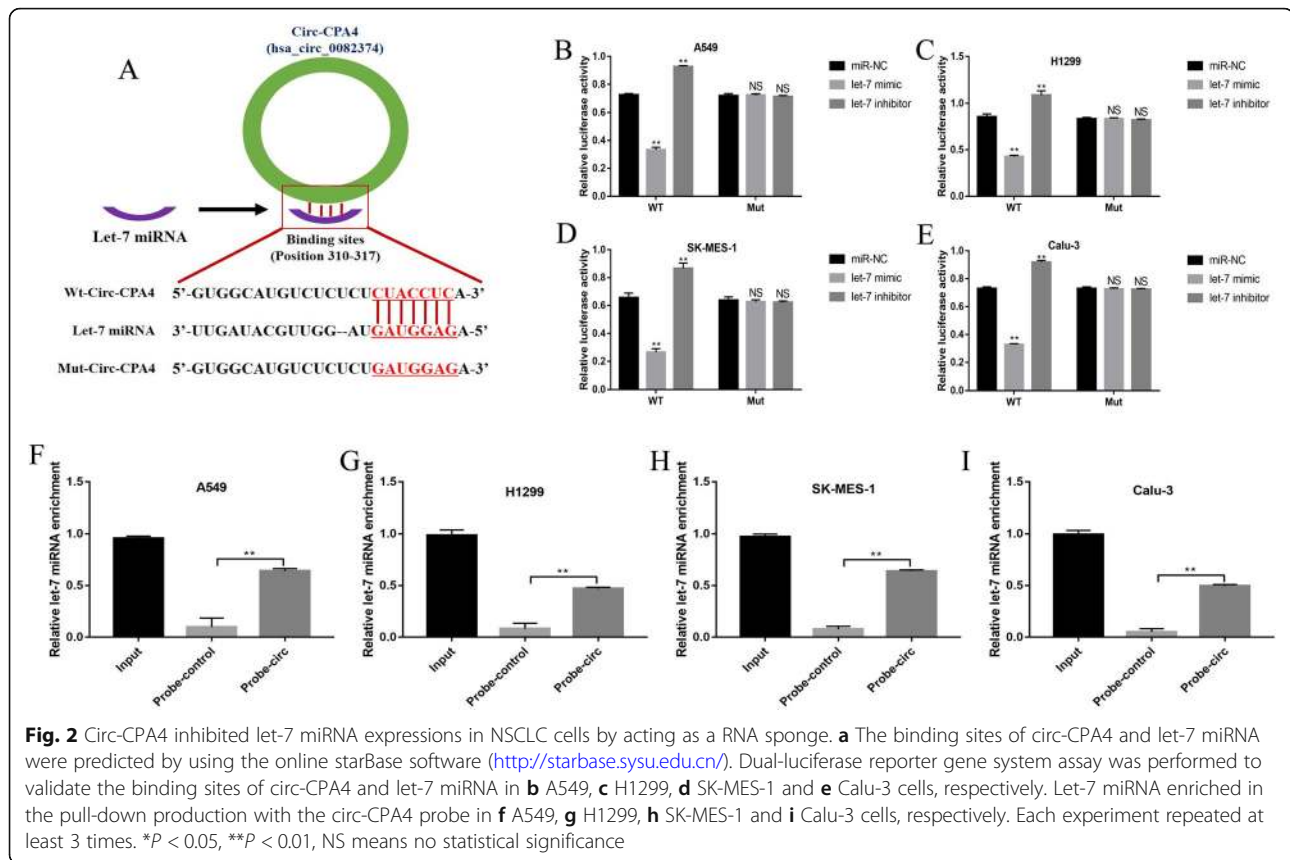
paired normal adjacent tissues (Fig. 1a-c). Further Western Blot results validated that PD-L1 protein was high-expressed in NSCLC tissues compared to the normal tissues (Fig. 1d, e). The above results were partly supported by the analysis by the data from The Cancer Genome Atlas (TCGA) database in lung squamous cell carcinoma (LUSC) (Fig. S4A-C). Besides, the levels of let-7 miRNA were negatively correlated with circ-CPA4 (Fig. 1f) and PD-L1 mRNA (Fig. 1g), and the levels of circ-CPA4 were positively correlated with PD-L1 mRNA (Fig. 1h) in NSCLC tissues. In addition, NSCLC patients with high-expressed circ-CPA4 and PD-L1, low-expressed let-7 miRNA tended to have a unfavorable prognosis (Fig. 1i-k). Specifically, the medium survival time was listed as follows: low-circ-CPA4 (34.5)/high-circ-CPA4 (18), low-let-7 miRNA (23)/high-let-7 miRNA (34) and low-PD-L1 (25)/high-PD-L1 (18). Also, the hazard ratio was listed as follows: Circ-CPA4 (A/B = 0.1184, B/A = 8.447), let-7 miRNA (A/B = 2.414, B/A = 0.4142) and PD-L1 (A/B = 0.3092, B/A = 3.234). However, the analysis from TCGA database indicated that circ-CPA4, let-7 miRNA and PD-L1 (CD274) were not correlated with patients prognosis in LUSC (Fig. S4D-F). Furthermore, the correlations of the expression levels of circ-CPA4, let-7 miRNA and PD-L1 mRNA, with the common clinicopathological characteristics were analyzed (Table 1-3). The results showed that circ-CPA4 and PD-L1 mRNA were high-expressed, while let-7 miRNA was low-expressed in NSCLC patients with high-grade TNM stage (III/IV) and lymphatic metastasis (Table 1-3). However, the above genes were not relevant to other clinical parameters, including patients age, gender, pathological type and smoking status (Table 1-3). Next, the in vitro experiments were conducted to determine the levels of circ-CPA4, let-7 miRNA and PD-L1 in NSCLC cell lines (A549, H1299, SK-MES-1 and Calu-3) and the normal HBE cells (Fig. 1l-p). The results showed that expression levels of circ-CPA4 (Fig. 1l) and PD-L1 (Fig. 1n, Fig. 1o, p) were higher, and let-7 miRNA (Fig. 1m) was lower in NSCLC cell lines compared to the HBE cells, which were in accordance with our clinical results.

Circular RNA circ-CPA4 served as a ceRNA and inhibited let-7 miRNA expressions in NSCLC cells

Previous study proved that let-7 miRNA was the downstream target of circ-CPA4 in glioma cells [34], hence we next explored whether circ-CPA4 could regulate let-7 miRNA levels in NSCLC cells. The online starBase software (<http://starbase.sysu.edu.cn/>) predicted the binding sites of circ-CPA4 and let-7 miRNA (Fig. 2a). The above targeting sites in circ-CPA4 were mutated and named as mutant circ-CPA4 (Mut-CPA4), and the corresponding wild-type circ-CPA4 was named as Wt-CPA4 (Fig. 2a). The dual-luciferase reporter gene system results showed that let-7 miRNA mimic significantly decreased the luciferase activity in both A549, H1299, SK-MES-1 and Calu-3 cells co-transfecting with Wt-CPA4 instead of Mut-CPA4, which were increased by co-transfecting cells with let-7 miRNA inhibitor (Fig. 2b-e). Consistently, the circ-CPA4 probe pull-down assay validated that let-7 miRNA could be sponged by circ-CPA4 in A549 (Fig. 2f), H1299 (Fig. 2g), SK-MES-1 (Fig. 2h) and Calu-3 (Fig. 2i) cells. In addition, the overexpression and downregulation vectors for circ-CPA4 were successfully delivered into the NSCLC cells (Fig. S2). The above results indicated that circ-CPA4 functioned as RNA sponges to inhibit let-7 miRNA in NSCLC cells, which were in line with the previous study [34].

PD-L1 was targeted and inhibited by overexpressing let-7 miRNA in NSCLC cells

The immuncheckpoint protein PD-L1 could be sponged and inhibited by let-7 miRNA in multiple cancers, which further enhanced the efficacy of cancer immunotherapy [29]. The online StarBase software (<http://starbase.sysu.edu.cn/>) predicted the binding sites of let-7 miRNA and the 3' untranslated region (3' UTR) of PD-L1 mRNA (Fig. 3a), and the dual-luciferase reporter gene system validated these binding sites (Fig. 3b-e). Specifically, let-7 miRNA mimic inhibited the luciferase activity in A549 (Fig. 3b), H1299 (Fig. 3c), SK-MES-1 (Fig. 3d) and Calu-3 (Fig. 3e) cells co-transfected with wild-type PD-L1 (Wt-PD-L1) vectors instead of mutant PD-L1 (Mut-PD-L1) vectors, which were



increased by co-transfecting cells with let-7 miRNA inhibitor (Fig. 3b-e). Similarly, the PD-L1 probe pull-down assay results verified that let-7 miRNA could be enriched by the probes of 3' UTR region of PD-L1 mRNA in NSCLC cells (Fig. 3f-i). In addition, the let-7 mimic and inhibitor were successfully delivered into the NSCLC cells (Fig. 3j), and the results showed that overexpressed let-7 miRNA significantly inhibited mRNA levels of PD-L1 in NSCLC cells, which were increased by transfecting cells with let-7 miRNA inhibitor (Fig. 3k). Consistently, let-7 miRNA overexpression also decreased the protein levels of PD-L1 in A549 and H1299 cells, which were increased by knocking down let-7 miRNA (Fig. 3l, m).

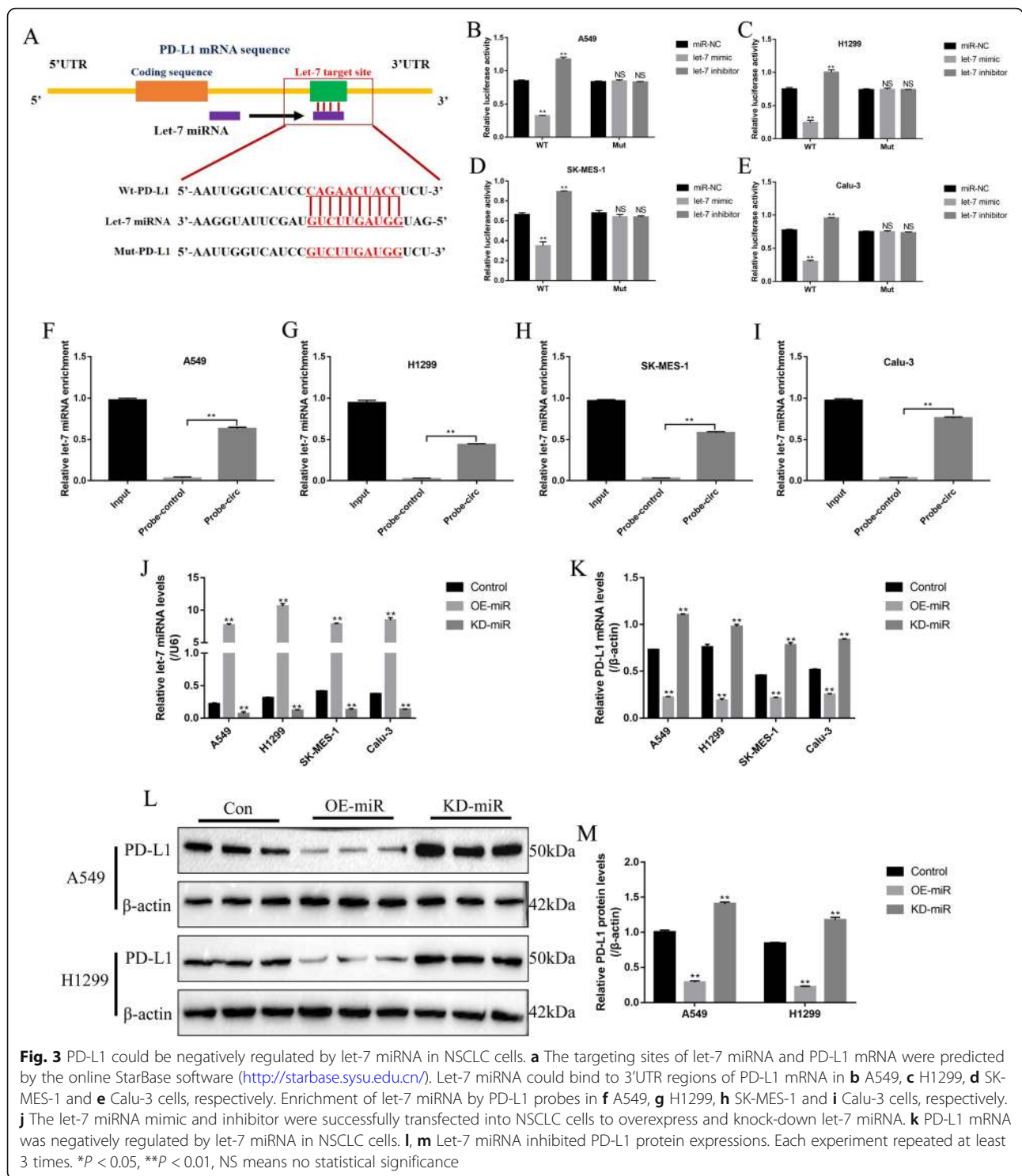
Circ-CPA4 downregulated let-7 miRNA to upregulate intracellular and extracellular PD-L1 in NSCLC cells

The above results enlightened us to speculate that circ-CPA4 might regulate PD-L1 levels in NSCLC cells by targeting let-7 miRNA. To validate the above hypothesis, the overexpression and downregulation vectors for circ-CPA4 were successfully delivered into NSCLC cell lines (Fig. S2). The Real-Time qPCR results showed that PD-L1 mRNA levels were increased by overexpressing circ-CPA4 and decreased by knocking down circ-CPA4 in NSCLC cells (A549, H1299, SK-MES-1 and Calu-3) (Fig.

4a-d), which were validated by the Western Blot assay at translational level in A549 and H1299 cells (Fig. 4e, f). Further results showed that the promoting effects of overexpressed circ-CPA4 on PD-L1 protein levels were abrogated by upregulating let-7 miRNA (Fig. 4g, h). In addition, the NSCLC cells derived exosomes were isolated from the supernatants, and analyzed by the Western Blot for TSG101 (Fig. S3) and PD-L1 expressions (Fig. 4i). The results showed that the exosome marker TSG101 was high-expressed in the isolated exosomes (Fig. S3), indicating that the exosomes were successfully purified by us. Also, we observed that PD-L1 protein existed in the exosomes (Fig. 4j, k). Of note, knock-down of circ-CPA4 decreased the expression levels of exosomal PD-L1 in the supernatants, which were restored by downregulating let-7 miRNA (Fig. 4j, k), suggesting that circ-CPA4 regulated intracellular and extracellular exosomal PD-L1 in NSCLC cells by sponging let-7 miRNA.

NSCLC cell derived exosomes self-regulated drug resistance and stemness in NSCLC cells through PD-L1

According to the previous study, cancer cells derived exosomes regulated drug resistance and stemness of cancer cells in breast cancer [42]. Thus, the effects of



NSCLC cell derived exosomes on NSCLC cell stemness and chemoresistance were investigated. By co-culturing the NSCLC cell derived exosomes with cisplatin-sensitive NSCLC cells, the PD-L1 levels in A549 and H1299 cells were significantly upregulated (Fig. 5a, b), suggesting that exosomal PD-L1 approached the

cytoplasm of the above NSCLC cells (Fig. 5c). Besides, the mRNA levels of the stemness associated signatures (OCT4, SOX2, Nanog and ALDH1) in NSCLC cells were all increased by exosomes treatment, compared to the control group, but anti-PD-L1 antibody abrogated the effects of NSCLC cell derived exosomes on cell

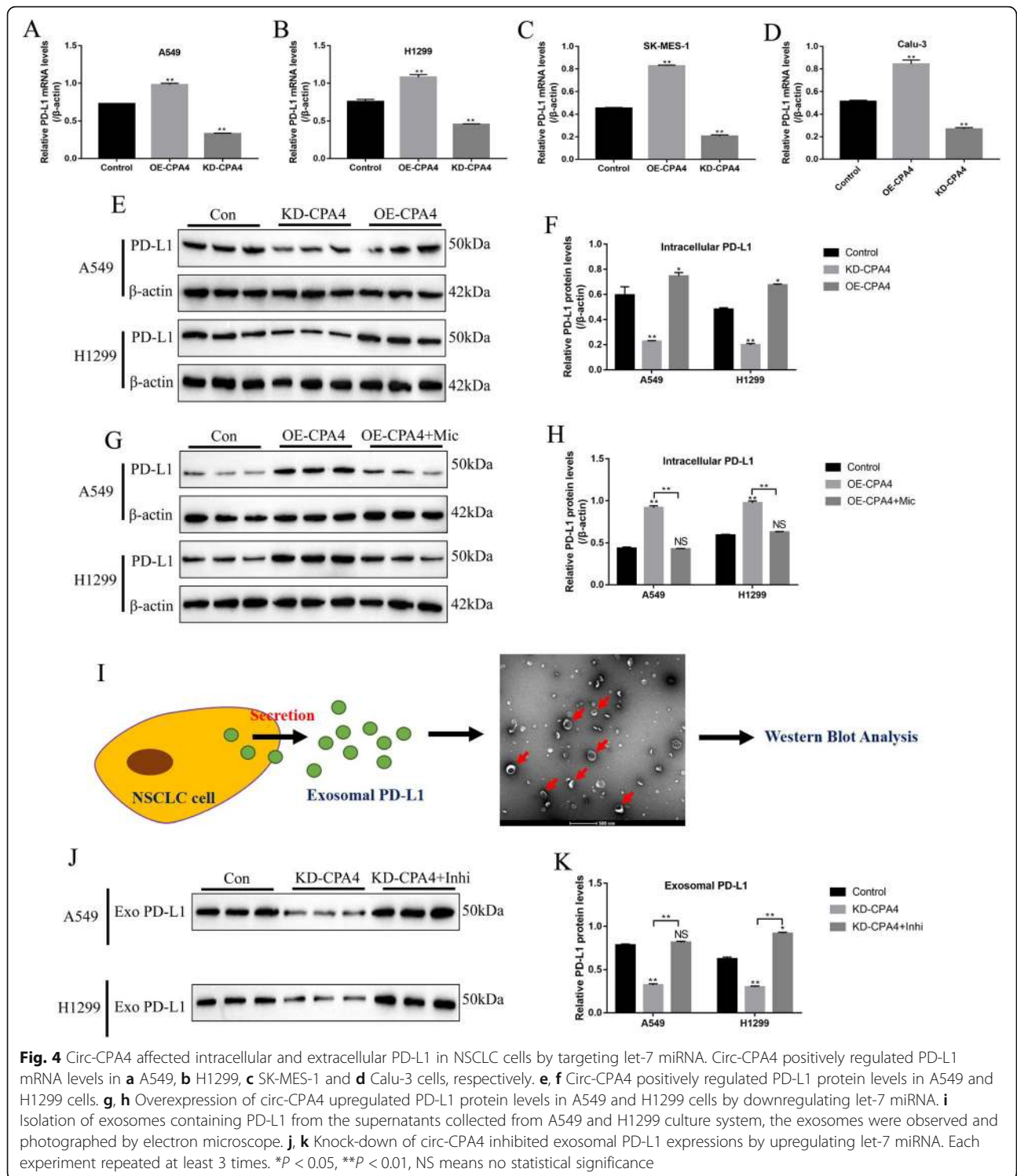


Fig. 4 Circ-CPA4 affected intracellular and extracellular PD-L1 in NSCLC cells by targeting let-7 miRNA. Circ-CPA4 positively regulated PD-L1 mRNA levels in **a** A549, **b** H1299, **c** SK-MES-1 and **d** Calu-3 cells, respectively. **e, f** Circ-CPA4 positively regulated PD-L1 protein levels in A549 and H1299 cells. **g, h** Overexpression of circ-CPA4 upregulated PD-L1 protein levels in A549 and H1299 cells by downregulating let-7 miRNA. **i** Isolation of exosomes containing PD-L1 from the supernatants collected from A549 and H1299 culture system, the exosomes were observed and photographed by electron microscope. **j, k** Knock-down of circ-CPA4 inhibited exosomal PD-L1 expressions by upregulating let-7 miRNA. Each experiment repeated at least 3 times. * $P < 0.05$, ** $P < 0.01$, NS means no statistical significance

stemness markers (Fig. 5d, e). In addition, the NSCLC cells were treated with high-dose cisplatin (20 μg/ml), and the NSCLC cells pretreated with NSCLC cell derived exosomes were more resistant to cisplatin treatment, which could be abrogated by co-treating cells with

anti-PD-L1 antibody (Fig. 5f-j). Specifically, high-dose cisplatin (20 μg/ml) inhibited cell viability (Fig. 5f, g) and promoted cell death (Fig. 5h-j) in A549 and H1299 cells, which were alleviated by pre-treating cells with NSCLC cell derived exosomes. Of note, anti-PD-L1 antibody

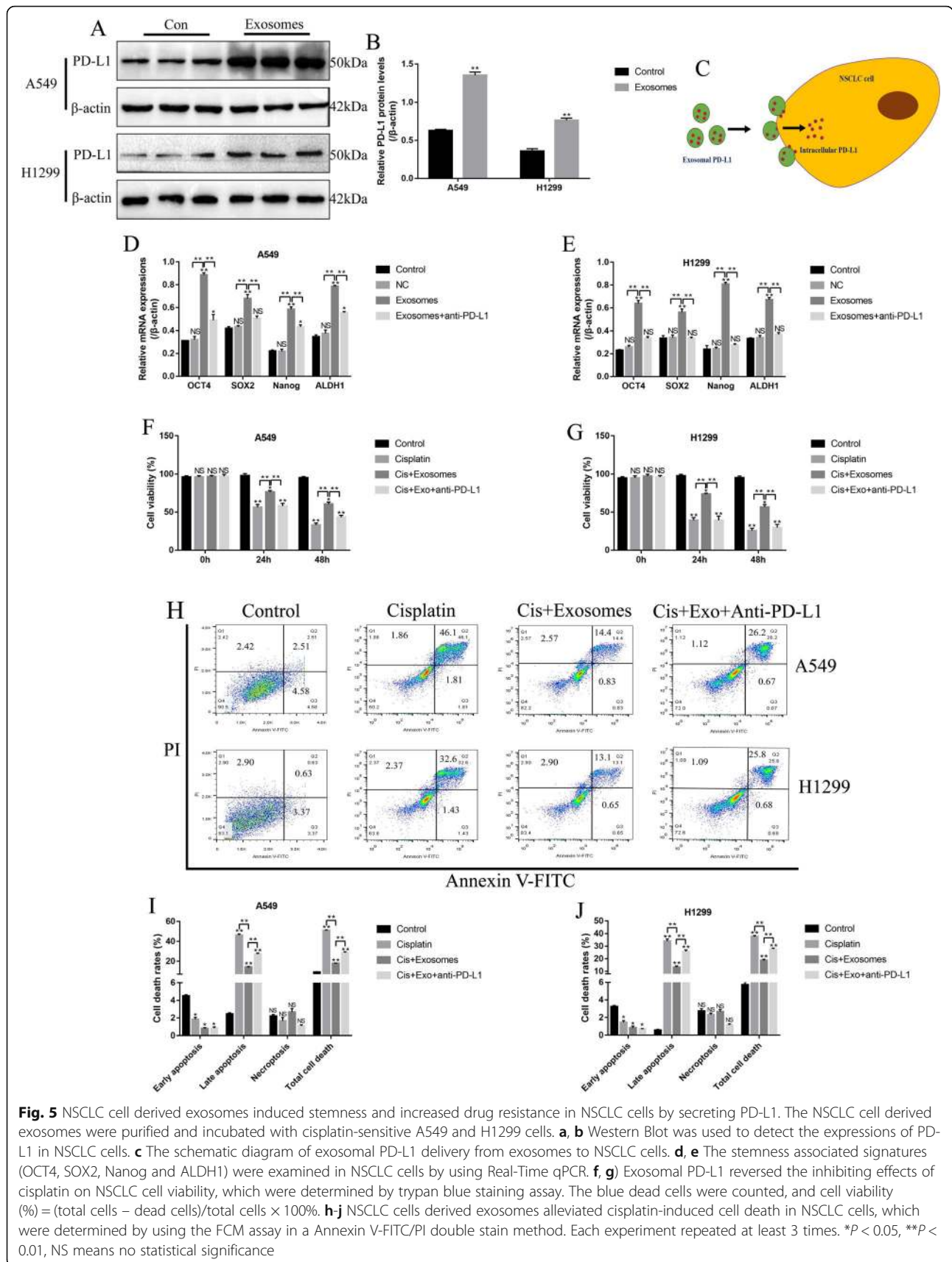


Fig. 5 NSCLC cell derived exosomes induced stemness and increased drug resistance in NSCLC cells by secreting PD-L1. The NSCLC cell derived exosomes were purified and incubated with cisplatin-sensitive A549 and H1299 cells. **a, b** Western Blot was used to detect the expressions of PD-L1 in NSCLC cells. **c** The schematic diagram of exosomal PD-L1 delivery from exosomes to NSCLC cells. **d, e** The stemness associated signatures (OCT4, SOX2, Nanog and ALDH1) were examined in NSCLC cells by using Real-Time qPCR. **f, g** Exosomal PD-L1 reversed the inhibiting effects of cisplatin on NSCLC cell viability, which were determined by trypan blue staining assay. The blue dead cells were counted, and cell viability (%) = (total cells – dead cells)/total cells \times 100%. **h-j** NSCLC cells derived exosomes alleviated cisplatin-induced cell death in NSCLC cells, which were determined by using the FCM assay in a Annexin V-FITC/PI double stain method. Each experiment repeated at least 3 times. * $P < 0.05$, ** $P < 0.01$, NS means no statistical significance

abrogated the protective effects of NSCLC cell derived exosomes on cisplatin-induced cell death in A549 and H1299 cells (Fig. 5f-j).

Knock-down of circ-CPA4 inhibited NSCLC cell growth by regulating let-7 miRNA/PD-L1 axis

Further experiments were conducted to investigate the effects of circ-CPA4/let-7 miRNA/PD-L1 axis on NSCLC cell proliferation and death. Knock-down of circ-CPA4 inhibited cell proliferation (Fig. 6a, b) and colony formation abilities (Fig. 6c, d) in A549 and H1299 cells, which were reversed by both knocking down let-7 miRNA or upregulating PD-L1 (Fig. 6a-d). Similarly, downregulation of circ-CPA4 inhibited cell viability (Fig. 6e, f) and promoted cell death (Fig. 6g-i) in NSCLC cells by upregulating let-7 miRNA and downregulating PD-L1. The above cellular results were also validated in vivo, and the results showed that knock-down of circ-CPA4 inhibited Cyclin D1 and Bcl-2 expressions, while increased the expression levels of Bax in mice tumor tissues, which were reversed by downregulating let-7 miRNA or upregulating PD-L1 (Fig. S1A-C). In addition, silencing of circ-CPA4 hampered tumorigenesis of NSCLC cells in xenograft tumor-bearing mice models by regulating let-7 miRNA/PD-L1 axis in a similar manner (Fig. S1D). The above results suggested that deficiency of circ-CPA4 inhibited NSCLC cell growth and promoted cell death in vitro and in vivo by targeting let-7 miRNA/PD-L1 axis.

The effects of circ-CPA4/let-7 miRNA/PD-L1 axis on NSCLC cell invasion and epithelial-mesenchymal transition (EMT)

We next explored whether circ-CPA4 participated in the regulation of NSCLC cell mobility and epithelial-mesenchymal transition (EMT) by regulating let-7 miRNA/PD-L1 axis (Fig. 7a-f). As expected, the transwell assay results showed that knock-down of circ-CPA4 inhibited the invasion ability of NSCLC cells (A549 and H1299), which were reversed by knocking down let-7 miRNA and upregulating PD-L1 (Fig. 7a, b). Furthermore, the expression levels of epithelial-mesenchymal transition (EMT) associated proteins (N-cadherin and Vimentin) were determined (Fig. 7c-f), and the results showed that knock-down of circ-CPA4 inhibited the expressions of N-cadherin and Vimentin, which were also increased by knocking down let-7 miRNA and upregulating PD-L1 in A549 and H1299 cells (Fig. 7c-f), indicating that circ-CPA4 regulated NSCLC cell mobility and EMT by targeting let-7 miRNA/PD-L1 axis.

NSCLC cells secreted PD-L1-containing exosomes to inactivate CD8⁺ T cells in the co-culturing system

Tumor cell derived extracellular PD-L1 promoted immune invasion in tumor immune microenvironment

by binding to its receptor PD-1 [39] and triggering intracellular cell death pathway [43], and this study investigate this issue by co-culturing the NSCLC cells with CD8⁺ T cells isolated from human peripheral blood mononuclear cells (hPBMCs) in a transwell co-culturing system to avoid direct cell-to-cell contact (Fig. 8a), which excluded the possibility that CD8⁺ T cells interacted with the tumor antigens presented in NSCLC cell membranes, but merely interacted with the secreting tumor antigens. Interestingly, CD8⁺ T cell viability was decreased in the co-culturing system, and silencing of PD-L1 in NSCLC cells or PD-L1/PD-1 blockade by their corresponding antibodies significantly promoted cell proliferation (Fig. 8b), viability (Fig. 8c) and inhibited cell death (Fig. 8d-f) in CD8⁺ T cells, suggesting that NSCLC cells secreted PD-L1 induced CD8⁺ T cell death in the co-culturing system. Furthermore, PD-L1 ablation also increased the expression levels of IFN- γ and IL-4, while inhibited IL-10 expressions in CD8⁺ T cells (Fig. 8g, h) and the supernatants (Fig. 8i, j), indicating that NSCLC cell inactivated CD8⁺ T cells in the co-culturing system through secreting PD-L1.

The NSCLC cells with downregulated circ-CPA4 activated CD8⁺ T cells in the co-culturing system

Further experiments were conducted to co-culture the NSCLC cells deficient or overexpression of circ-CPA4 with CD8⁺ T cells in the co-culturing system. The results showed that NSCLC cells with downregulated circ-CPA4 significantly increased the proliferation ability (Fig. 9a) and viability (Fig. 9b), and inhibited cell apoptosis (Fig. 9c-e) of CD8⁺ T cells in the co-culturing system, while overexpression of circ-CPA4 had opposite effects on the above cell functions (Fig. 9a-e). Consistently, NSCLC cells with circ-CPA4 overexpression promoted Bax expressions in CD8⁺ T cells, and NSCLC cells with downregulated circ-CPA4 had opposite effects on Bax expressions (Fig. 9f, g). In addition, silencing of circ-CPA4 in NSCLC cells activated CD8⁺ T cells in the co-culturing system (Fig. 9h-k). Specifically, NSCLC cells with circ-CPA4 downregulation increased the levels of IFN- γ and IL-4, and inhibited IL-10 expressions in CD8⁺ T cells (Fig. 9h, i) and the supernatants (Fig. 9j, k) of the co-culture system. Conversely, overexpression of circ-CPA4 in NSCLC cells inactivated CD8⁺ T cells in the co-culturing system (Fig. 9h-k). The above results suggested that knock-down of circ-CPA4 in NSCLC cells promoted CD8⁺ T cell expansion and activation in the co-culturing system.

Discussion

Previous data suggested that circ-CPA4 [34] and PD-L1 [22] served as oncogenes, while let-7 miRNA acted as tumor suppressor [28] in cancer development, and there

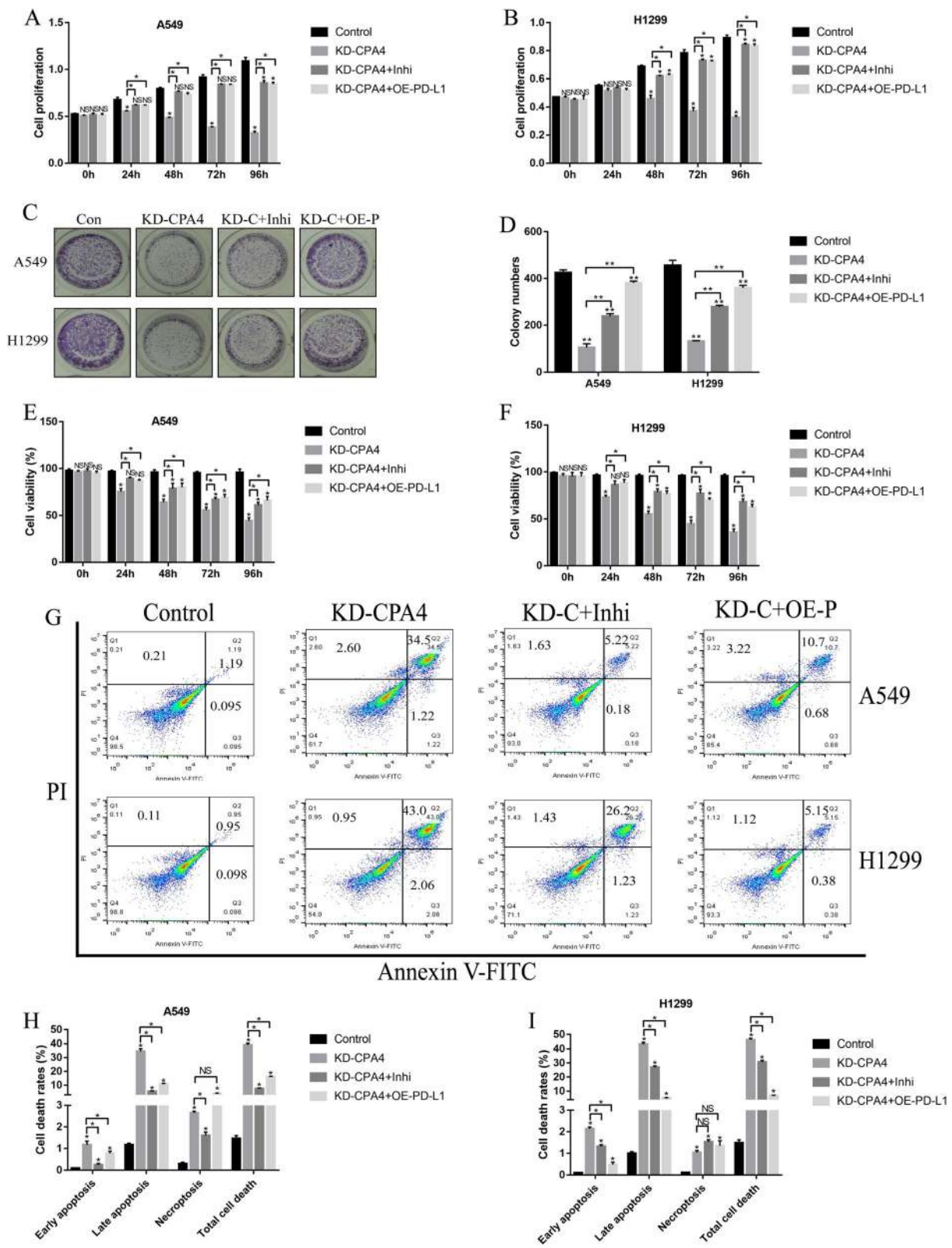


Fig. 6 (See legend on next page.)

(See figure on previous page.)

Fig. 6 Circ-CPA4 regulated NSCLC cell proliferation and death by targeting let-7 miRNA/PD-L1 axis in vitro and in vivo. Knock-down of circ-CPA4 inhibited cell proliferation in **a** A549 and **b** H1299 cells by upregulating let-7 miRNA and downregulating PD-L1. **c, d** The colony formation abilities were inhibited by knocking down circ-CPA4 through let-7 miRNA/PD-L1 axis. **e, f** Trypan blue staining assay was performed to evaluate cell viability in NSCLC cells. **g-i** The apoptosis ratio of NSCLC cells were determined by using the FCM assay. Each experiment repeated at least 3 times. * $P < 0.05$, ** $P < 0.01$, NS means no statistical significance

existed potential regulating mechanisms among circ-CPA4, let-7 miRNA and PD-L1 in cancer cells [29, 34], which were validated in this study in NSCLC cells. Specifically, by analyzing the clinical samples and cell lines, circ-CPA4 and PD-L1 were high-expressed, while let-7 miRNA was low-expressed in malignant tissues and cells compared to their paired normal counterparts. Also, NSCLC patients with lower levels of circ-CPA4 and PD-L1, and higher levels of let-7 miRNA tended to have a better prognosis. The above results were partly evidenced by the Pan-Cancer analysis based on the TCGA database, which showed that circ-CPA4 was high-expressed, while let-7 miRNA was low-expressed in

cancer tissues, compared to their adjacent normal tissues, in lung squamous cell carcinoma (LUSC). However, the expression levels of PD-L1 (CD274) were not changed in LUSC tissues. In addition, the correlations of circ-CPA4/let-7 miRNA/PD-L1 pathway with patients prognosis were not significant. The reason for the above phenomena could be attributed to the poor sample size and limited cancer types, resulting in possible systemic and random errors or bias. Thus, our further work will enlarge the sample size and include multiple cancer types to solve this problem. Next, based on the previous studies [29, 34], we also uncovered the regulating mechanisms of circ-CPA4, let-7 miRNA and

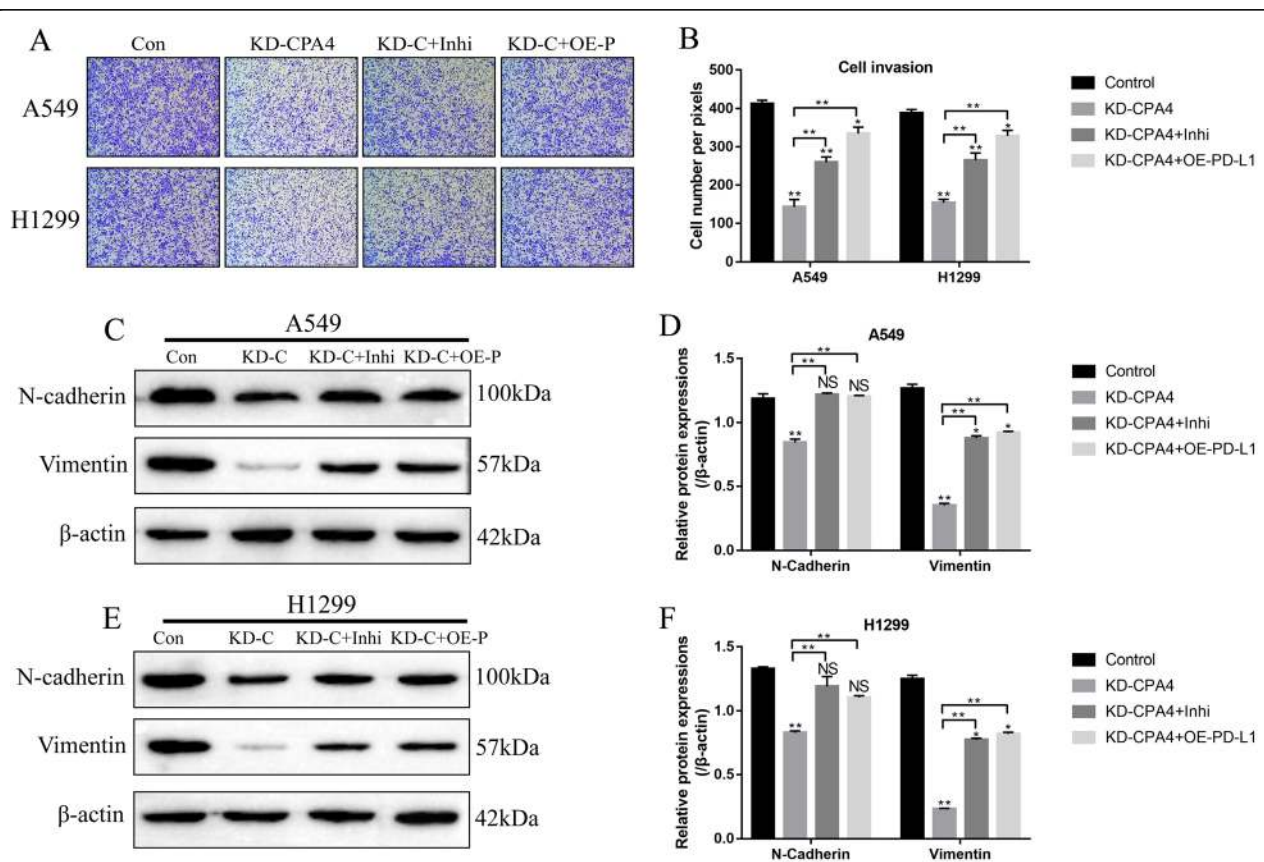
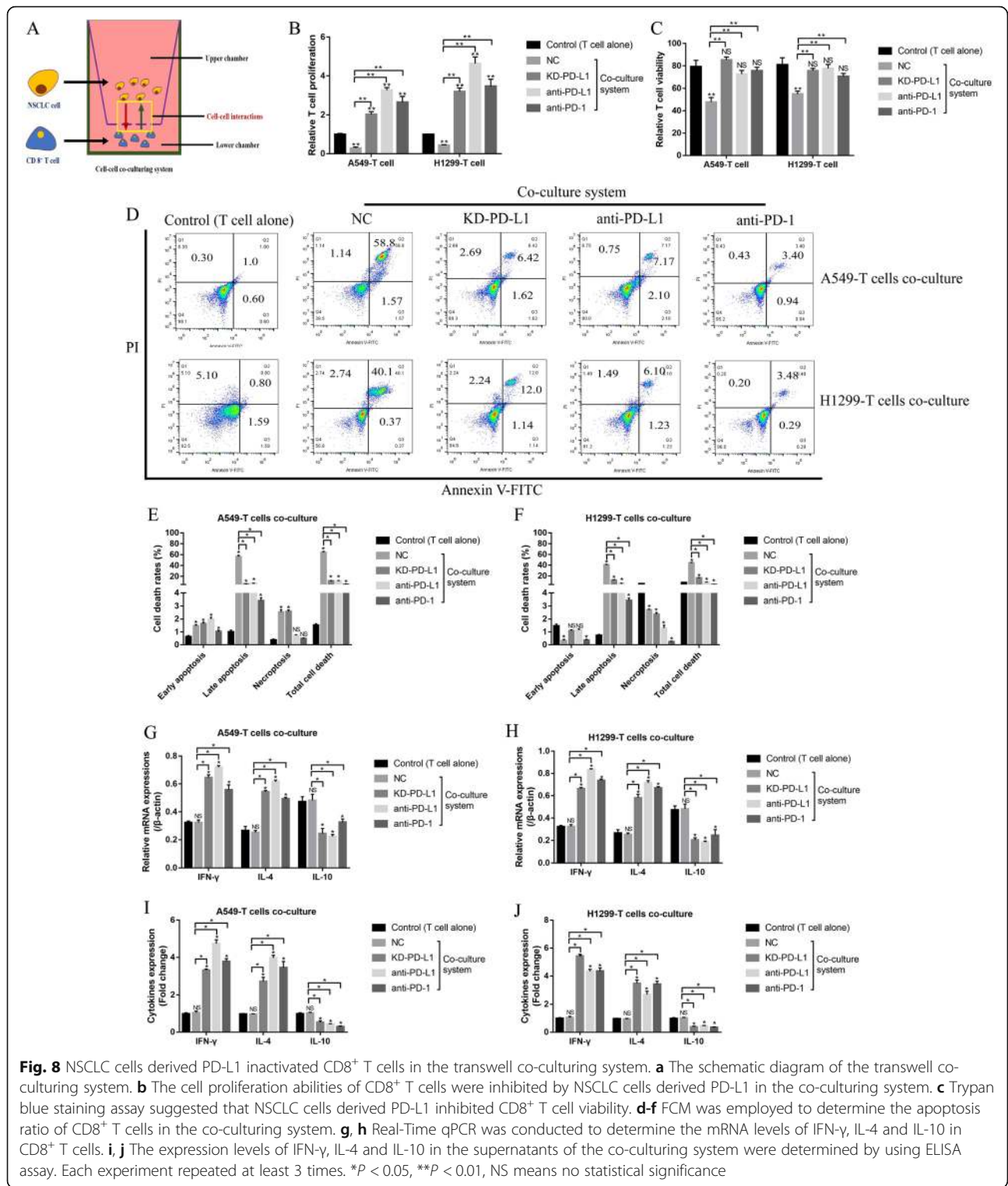


Fig. 7 Circ-CPA4/let-7 miRNA/PD-L1 axis regulated NSCLC cell invasion, migration and epithelial-mesenchymal transition (EMT) in vitro. **a, b** Knock-down of circ-CPA4 inhibited NSCLC cell invasion by targeting let-7 miRNA and PD-L1. **c-f** Circ-CPA4 silencing inhibited the expression levels of EMT associated proteins (N-cadherin and Vimentin), which were abrogated by downregulating let-7 miRNA and upregulating PD-L1. The protein levels were determined by using Western Blot analysis. Each experiment repeated at least 3 times. * $P < 0.05$, ** $P < 0.01$, NS means no statistical significance



PD-L1 in NSCLC cells, and found that circ-CPA4 overexpression promoted PD-L1 expressions by acting as RNA sponges for let-7 miRNA. Furthermore, the gain-and-loss of function experiments validated that

knock-down of circ-CPA4 inhibited NSCLC cell growth, mobility, EMT and tumorigenesis, and promoted cell death by regulating let-7 miRNA/PD-L1 axis in vitro and in vivo. The above results were in accordance with

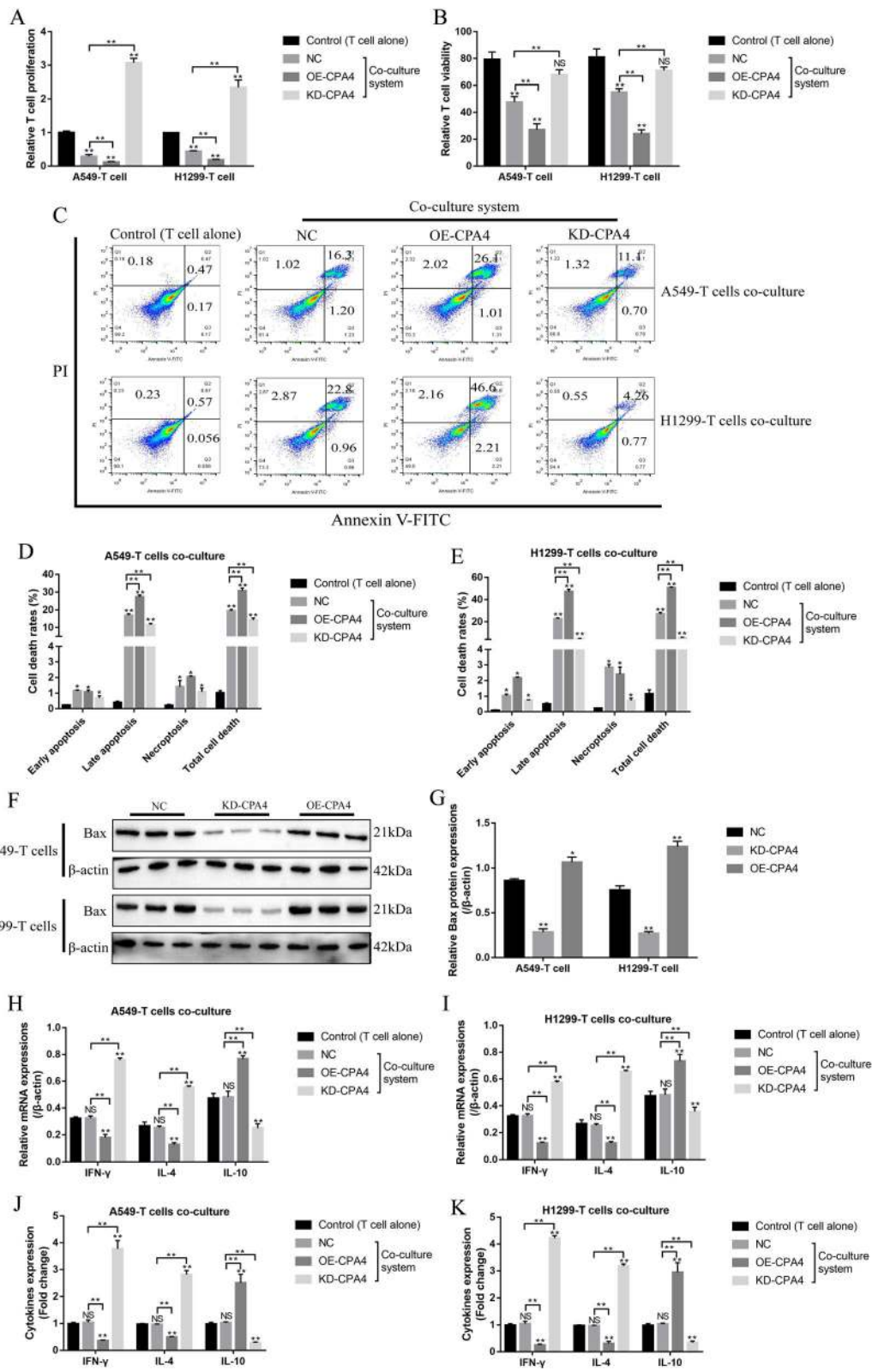


Fig. 9 (See legend on next page.)

(See figure on previous page.)

Fig. 9 NSCLC cells with differential circ-CPA4 expressions influenced CD8⁺ T cell expansion and activation in the co-culturing system. **a** Knock-down of circ-CPA4 promoted cell proliferation in CD8⁺ T cells, while circ-CPA4 overexpression had opposite effects. **b** The effects of NSCLC cells differential circ-CPA4 expressions on CD8⁺ T cell viability were determined by trypan blue staining assay. **c-e** FCM was conducted to examine the apoptosis ratio of CD8⁺ T cells in the co-culturing system. **f-g** Western Blot was performed to detect the expression levels of Bax in CD8⁺ T cells co-cultured with NSCLC cells. **h, i** Real-Time qPCR was conducted to detect the mRNA levels of inflammatory cytokines (IFN- γ , IL-4 and IL-10) in CD8⁺ T cells. **j, k** The expression status of IFN- γ , IL-4 and IL-10 in the supernatants were measured by ELISA. Each experiment repeated at least 3 times. * $P < 0.05$, ** $P < 0.01$, NS means no statistical significance

the previous data [22, 28], and indicated that knock-down of circ-CPA4 inhibited NSCLC progression by inhibiting PD-L1 through releasing let-7 miRNA.

Currently, resistance of NSCLC cells to chemotherapeutic drugs seriously limited their therapeutic efficacy in clinic [44, 45], and discovery of novel therapeutic agents to improve the sensitivity of chemoresistant NSCLC cells became urgent. Previous data indicated that cancer cells derived PD-L1-containing exosomes contributed a lot to drug resistance by inducing cancer stem cells (CSCs) to sustain the heterogeneous lineages of cancer cells in response to chemotherapy [42]. Mechanistically, the CSCs differentiated into heterogeneous cells, and the chemoresistant cancer cells survived and expanded under the long-term chemotherapeutic drugs stimulation [42]. As a result, the survival cancer cells were resistant to the drugs [46, 47]. Interestingly, the above results were also validated in NSCLC cells, and we found that NSCLC cells derived exosomes increased resistance of NSCLC cells to cisplatin treatment and up-regulated stemness associated signatures (OCT4, SOX2, Nanog and ALDH1), which were all abrogated by treating cells with anti-PD-L1 antibody, indicating that NSCLC cells derived PD-L1 exosomes self-regulated cell stemness to increase resistance of NSCLC cells to cisplatin, and blockade of PD-L1 sensitized chemoresistant NSCLC cells to cisplatin, which were in accordance with the previous data [42].

Disabled immune surveillance was found in NSCLC [1, 2], which contributed to immune evasion of NSCLC cells [3]. Most studies confirmed that T cells, especially CD8⁺ T cells, were crucial for tumor elimination by recognizing tumor specific antigens [48, 49]. However, recent data suggested that cancer cells derived PD-L1 inactivated infiltrating CD8⁺ T cells in the tumor microenvironment by activating PD-1 mediated extracellular pathway [19] and NLRP3 mediated intracellular pathway [50] mediated cell death, but the detailed mechanisms are still unclear. By co-culturing the NSCLC cells with CD8⁺ T cells isolated from human peripheral blood mononuclear cells (hPBMCs) in a transwell co-culturing system to avoid direct cell-to-cell contact, we found that NSCLC cells inhibited cell expansion, viability and pro-inflammatory cytokines secretion, while promoted cell death in CD8⁺ T cells in a secreted PD-L1 dependent

manner, suggesting that NSCLC cells inactivated CD8⁺ T cells by secreting PD-L1 in the in vitro co-culturing system. Interestingly, we found that circ-CPA4 also positively regulated exosomal PD-L1 expressions by silencing let-7 miRNA in the supernatants of NSCLC cells, hence the NSCLC cells deficient of circ-CPA4 were next co-cultured with CD8⁺ T cells. As expected, the NSCLC cells with circ-CPA4 ablation increased CD8⁺ T cell proliferation and activity, but circ-CPA4 overexpression had opposite effects. The above in vitro results indicated that knock-down of circ-CPA4 in NSCLC cells activated CD8⁺ T cells in tumor microenvironment by downregulating secreted PD-L1.

Conclusions

Collectively, this study found that knock-down of circ-CPA4 inhibited intracellular and extracellular PD-L1 by targeting let-7 miRNA. On the one hand, PD-L1 self-regulated NSCLC cell growth, mobility, stemness and chemoresistance to cisplatin treatment. On the other, secreted PD-L1 inactivated CD8⁺ T cells by activating extracellular and intracellular pathways mediated cell death to facilitate immune evasion. However, future experiments are still needed to investigate the detailed mechanisms of PD-L1 induced CD8⁺ T cell death and inactivation.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s13046-020-01648-1>.

Additional file 1 Figure S1. The A549 and H1299 cells transfected with different vectors were used to generate xenograft mice models, the tumor tissues were collected and (A-C) Western Blot was used to examine the expression levels of Cyclin D1, Bax and Bcl-2 in tumor tissues. (D) Tumors were photographed and tumor weight was measured to evaluate tumorigenesis of NSCLC cells in vivo. * $P < 0.05$.

Additional file 2 Figure S2. The overexpression and downregulation vectors for circ-CPA4 were successfully delivered into NSCLC cells (A549, H1299, SK-MES-1 and Calu-3), examined by using the Real-Time qPCR. *** $P < 0.01$.

Additional file 3 Figure S3. Western Blot analysis was performed to examine the expression levels of TSG101 in the isolated exosomes.

Additional file 4 Figure S4. The Pan-cancer analysis was conducted by using the online starBase software (<http://starbase.sysu.edu.cn/panCancer.php>), and the (A-C) expression levels and (D-F) prognosis relevance of circ-CPA4, let-7 miRNA and PD-L1 in lung squamous cell carcinoma (LUSC) were analyzed.

Abbreviations

NSCLC: Non-small cell lung cancer; PD-L1: Programmed cell death ligand 1; HBE: Human bronchial epithelial cells; EMT: Epithelial-mesenchymal transition; hPBMCs: Human peripheral blood mononuclear cells; miRNAs: microRNAs; 3'UTR: 3' untranslated region; circRNAs: Circular RNAs; ceRNA: Competing endogenous RNA; ECL: Electrochemiluminescence; PBS: Phosphate buffer saline; CCK-8: Cell counting kit-8; OD: Optical density; FCM: Flow cytometry; PI: Propidium iodide; ELISA: Enzyme linked immunosorbent assay; SD: Standard deviation; CSCs: Cancer stem cells

Acknowledgements

Not applicable.

Authors' contributions

Dr. Weijun Hong and Min Xue designed and conducted most of the experiments in this study, besides, they drafted the manuscript and contributed equally to this work. Dr. Jun Jiang and Yajuan Zhang finished the rest part of the experiments in this study, and they analyzed the data and designed the figures. Dr. Xiwen Gao and Yajuan Zhang provided guidance for this work and revised the manuscript, they contributed equally to this work. In addition, Dr. Xiwen Gao financially supported this work and responsible for manuscript submission. The author(s) read and approved the final manuscript.

Funding

This study was financially supported by the Health and Family planning Commission of Shanghai (Grant No. 201940102) and Youth Fund of Minhang Hospital Fudan University (Grant No. 2019MHJC08).

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

All the clinical experiments in this study were approved by the Ethics Committee of Minhang Hospital, Fudan University. Besides, the written informed consent was obtained from all the participants involved in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Department of Respiratory Medicine, Minhang Hospital, Fudan University, 170 Xin-Song Road, Shanghai 201199, China. ²State Key Laboratory of Genetic Engineering, Shanghai Engineering Research Center of Industrial Microorganisms, School of Life Sciences, Fudan University, Shanghai 200438, China.

Received: 22 April 2020 Accepted: 21 July 2020

Published online: 03 August 2020

References

- Lin A, Wei T, Meng H, Luo P, Zhang J. Role of the dynamic tumor microenvironment in controversies regarding immune checkpoint inhibitors for the treatment of non-small cell lung cancer (NSCLC) with EGFR mutations. *Mol Cancer*. 2019;18(1):139.
- Santaniello A, Napolitano F, Seretto A, De Placido P, Silvestris N, Bianco C, et al. Tumour Microenvironment and Immune Evasion in EGFR Addicted NSCLC: Hurdles and Possibilities. *Cancers (Basel)*. 2019;11(10).
- Li, Y.M., J.M. Yu, Z.Y. Liu, H.J. Yang, J. Tang, and Z.N. Chen. Programmed Death Ligand 1 Indicates Pre-Existing Adaptive Immune Response by Tumor-Infiltrating CD8(+) T Cells in Non-Small Cell Lung Cancer. *Int J Mol Sci*, 2019. 20(20).
- Rolfo C, Caglevic C, Santarpia M, Araujo A, Giovannetti E, Gallardo CD, et al. Immunotherapy in NSCLC: a promising and revolutionary weapon. *Adv Exp Med Biol*. 2017;995:97–125.
- Sgambato A, Casaluze F, Sacco PC, Palazzolo G, Maione P, Rossi A, et al. Anti PD-1 and PDL-1 immunotherapy in the treatment of advanced non-small cell lung cancer (NSCLC): a review on toxicity profile and its management. *Curr Drug Saf*. 2016;11(1):62–8.
- Luo F, Luo M, Rong QX, Zhang H, Chen Z, Wang F, et al. Niclosamide, an antihelmintic drug, enhances efficacy of PD-1/PD-L1 immune checkpoint blockade in non-small cell lung cancer. *J Immunother Cancer*. 2019;7(1):245.
- Sun NY, Chen YL, Wu WY, Lin HW, Chiang YC, Chang CF, et al. Blockade of PD-L1 Enhances Cancer Immunotherapy by Regulating Dendritic Cell Maturation and Macrophage Polarization. *Cancers (Basel)*. 2019;11(9).
- Arias Ron D, Labandeira CM, Areses Manrique MC, Sampedro Domarco P, Abdulkader I, Garcia-Mata J, et al. Dramatic Response of Leptomeningeal Carcinomatosis to Nivolumab in PD-L1 Highly Expressive Non-small Cell Lung Cancer: A Case Report. *Front Oncol*. 2019;9:819.
- Hellmann MD, Paz-Ares L, Bernabe Caro R, Zurawski B, Kim SW, Carcereny Costa E, et al. Nivolumab plus Ipilimumab in advanced non-small-cell lung cancer. *N Engl J Med*. 2019.
- Reck M, Rodriguez-Abreu D, Robinson AG, Hui R, Czoszi T, Fulop A, et al. Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. *N Engl J Med*. 2016;375(19):1823–33.
- Peters S, Kerr KM, Stahel R. PD-1 blockade in advanced NSCLC: a focus on pembrolizumab. *Cancer Treat Rev*. 2018;62:39–49.
- Santini FC, Rudin CM. Atezolizumab for the treatment of non-small cell lung cancer. *Expert Rev Clin Pharmacol*. 2017;10(9):935–45.
- Antonia SJ, Villegas A, Daniel D, Vicente D, Murakami S, Hui R, et al. Durvalumab after Chemoradiotherapy in stage III non-small-cell lung cancer. *N Engl J Med*. 2017;377(20):1919–29.
- Rosell R, Karachaliou N. Avelumab in non-small-cell lung cancer. *Lancet Oncol*. 2018;19(11):1423–4.
- Alvarez-Sierra D, Marin-Sanchez A, Ruiz-Blazquez P, de Jesus Gil C, Iglesias-Felip C, Gonzalez O, et al. Analysis of the PD-1/PD-L1 axis in human autoimmune thyroid disease: insights into pathogenesis and clues to immunotherapy associated thyroid autoimmunity. *J Autoimmun*. 2019;103:102285.
- Liu J, Yuan Y, Chen W, Putra J, Suriawinata AA, Schenk AD, et al. Immune-checkpoint proteins VISTA and PD-1 nonredundantly regulate murine T-cell responses. *Proc Natl Acad Sci U S A*. 2015;112(21):6682–7.
- El-Zaatari M, S. Bishu, M. Zhang, H. Grasberger, G. Hou, H.R. Haley, et al. Aim2-mediated/IFN-beta-independent regulation of gastric Metaplastic lesions via CD8+ T cells. *JCI Insight*, 2020.
- Hurkmans DP, Kuipers ME, Smit J, van Marion R, Mathijssen RHJ, Postmus PE, et al. Tumor mutational load, CD8(+) T cells, expression of PD-L1 and HLA class I to guide immunotherapy decisions in NSCLC patients. *Cancer Immunol Immunother*. 2020.
- Juneja VR, McGuire KA, Manguso RT, LaFleur MW, Collins N, Haining WN, et al. PD-L1 on tumor cells is sufficient for immune evasion in immunogenic tumors and inhibits CD8 T cell cytotoxicity. *J Exp Med*. 2017; 214(4):895–904.
- Del Re M, Marconcini R, Pasquini G, Rofi E, Vivaldi C, Bloise F, et al. PD-L1 mRNA expression in plasma-derived exosomes is associated with response to anti-PD-1 antibodies in melanoma and NSCLC. *Br J Cancer*. 2018;118(6): 820–4.
- Li C, Li C, Zhi C, Liang W, Wang X, Chen X, et al. Clinical significance of PD-L1 expression in serum-derived exosomes in NSCLC patients. *J Transl Med*. 2019;17(1):355.
- He PX, Ma ZL, Han H, Zhang XY, Niu SH, Du LN, et al. Expression of programmed death ligand 1 (PD-L1) is associated with metastasis and differentiation in gastric cancer. *Life Sci*. 2020;242:117247.
- Gao L, Guo Q, Li X, Yang X, Ni H, Wang T, et al. Corrigendum to 'MiR-873/PD-L1 axis regulates the stemness of breast cancer cells' *EBioMedicine* 41 (2019) 395–407. *EBioMed*. 2019;49:389–90.
- Li J, Chen M, Yu B. miR-433 suppresses tumor progression via Smad2 in non-small cell lung cancer. *Pathol Res Pract*. 2019;215(10):152591.
- Yang YN, Bian LQ, Ling XD, Fang CY, Jiang SL. MicroRNA-421 promotes proliferation and invasion of non-small cell lung cancer cells through targeting PDCCD4. *Pathol Res Pract*. 2019;215(10):152555.
- Biamonte F, Santamaria G, Sacco A, Perrone FM, Di Cello A, Battaglia AM, et al. MicroRNA let-7g acts as tumor suppressor and predictive biomarker for chemoresistance in human epithelial ovarian cancer. *Sci Rep*. 2019;9(1):5668.

27. Qin MM, Chai X, Huang HB, Feng G, Li XN, Zhang J, et al. BMC Urol. 2019; 19(1):–53.
28. Tsai CH, Lin LT, Wang CY, Chiu YW, Chou YT, Chiu SJ, et al. Over-expression of cofilin-1 suppressed growth and invasion of cancer cells is associated with up-regulation of let-7 microRNA. *Biochim Biophys Acta*. 2015;1852(5): 851–61.
29. Chen Y, Xie C, Zheng X, Nie X, Wang Z, Liu H, et al. LIN28/let-7/PD-L1 pathway as a target for Cancer immunotherapy. *Cancer Immunol Res*. 2019; 7(3):487–97.
30. Gilles ME, Slack FJ. Let-7 microRNA as a potential therapeutic target with implications for immunotherapy. *Expert Opin Ther Targets*. 2018; 22(11):929–39.
31. Dou R, Nishihara R, Cao Y, Hamada T, Mira K, Masuda A, et al. MicroRNA let-7, T cells, and patient survival in colorectal Cancer. *Cancer Immunol Res*. 2016;4(11):927–35.
32. Wang T, Wang X, Du Q, Wu N, Liu X, Chen Y, et al. The circRNA circP4HB promotes NSCLC aggressiveness and metastasis by sponging miR-133a-5p. *Biochem Biophys Res Commun*. 2019;513(4):904–11.
33. Liu G, Shi H, Deng L, Zheng H, Kong W, Wen X, et al. Circular RNA circ-FOXM1 facilitates cell progression as ceRNA to target PDPDF and MACC1 by sponging miR-1304-5p in non-small cell lung cancer. *Biochem Biophys Res Commun*. 2019;513(1):207–12.
34. Peng H, Qin C, Zhang C, Su J, Xiao Q, Xiao Y, Xiao K, Liu Q. circCPA4 acts as a prognostic factor and regulates the proliferation and metastasis of glioma. *J Cell Mol Med*. 2019;23(10):6658–65.
35. Jia N, Tong H, Zhang Y, Katayama H, Wang Y, Lu W, et al. CeRNA expression profiling identifies KIT-related circRNA-miRNA-mRNA networks in gastrointestinal stromal tumour. *Front Genet*. 2019;10:825.
36. Liang Y, Zhang Y, Xu L, Zhou D, Jin Z, Zhou H, et al. CircRNA Expression Pattern and ceRNA and miRNA-mRNA Networks Involved in Anther Development in the CMS Line of *Brassica campestris*. *Int J Mol Sci*. 2019; 20(19).
37. Zhu J, Zhang X, Gao W, Hu H, Wang X, Hao D. lncRNA/circRNAmiRNAmRNA ceRNA network in lumbar intervertebral disc degeneration. *Mol Med Rep*. 2019;20(4):3160–74.
38. Zhao R, Song Y, Wang Y, Huang Y, Li Z, Cui Y, et al. PD-1/PD-L1 blockade rescue exhausted CD8+ T cells in gastrointestinal stromal tumours via the PI3K/Akt/mTOR signalling pathway. *Cell Prolif*. 2019;52(3):e12571–e12571.
39. Chen G, Huang AC, Zhang W, Zhang G, Wu M, Xu W, et al. Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response. *Nature*. 2018;560(7718):382–6.
40. Zhou W-Y, Zhang M-M, Liu C, Kang Y, Wang J-O, Yang X-H. Long noncoding RNA LINC00473 drives the progression of pancreatic cancer via upregulating programmed death-ligand 1 by sponging microRNA-195-5p. *J Cell Physiol*. 2019;234(12):23176–89.
41. Wang X, He Q, Shen H, Xia A, Tian W, Yu W, et al. TOX promotes the exhaustion of antitumor CD8(+) T cells by preventing PD1 degradation in hepatocellular carcinoma. *J Hepatol*. 2019;71(4):731–41.
42. Gao L, Guo Q, Li X, Yang X, Ni H, Wang T, et al. MiR-873/PD-L1 axis regulates the stemness of breast cancer cells. *EBioMed*. 2019;41:395–407.
43. Theivanthiran B, Evans KS, DeVito NC, Plebanek M, Sturdivant M, Wachsmuth LP, Salama AK, Kang Y, Hsu D, Balko JM, Johnson DB, Starr M, Nixon AB, Holtzhausen A, Hanks BA. A tumor-intrinsic PD-L1-NLRP3 inflammasome signaling pathway drives resistance to anti-PD-1 immunotherapy. *J Clin Invest*. 2020;130(5):2570–86.
44. Ji L, Zhang R, Chen J, Xue Q, Moghal N, Tsao M-S. PIDD interaction with KEAP1 as a new mutation-independent mechanism to promote NRF2 stabilization and chemoresistance in NSCLC. *Sci Rep*. 2019;9(1):12437.
45. Wang X, Meng Q, Qiao W, Ma R, Ju W, Hu J, et al. miR-181b/Notch2 overcome chemoresistance by regulating cancer stem cell-like properties in NSCLC. *Stem Cell Res Ther*. 2018;9(1):327.
46. Su S, Chen J, Yao H, Liu J, Yu S, Lao L, et al. CD10(+)GPR77(+) Cancer-Associated Fibroblasts Promote Cancer Formation and Chemoresistance by Sustaining Cancer Stemness. *Cell*. 2018;172(4):841–56 e16.
47. Wang T, Fahrman JF, Lee H, Li Y-J, Tripathi SC, Yue C, et al. JAK/STAT3-Regulated Fatty Acid β -Oxidation Is Critical for Breast Cancer Stem Cell Self-Renewal and Chemoresistance. *Cell Metab*. 2018;27(1):136–50 e5.
48. Fu C, Jiang A. Dendritic cells and CD8 T cell immunity in tumor Microenvironment. *Front Immunol*. 2018;9:3059.
49. Horton BL, Williams JB, Cabanov A, Spranger S, Gajewski TF. Intratumoral CD8(+) T-cell apoptosis is a major component of T-cell dysfunction and impedes antitumor immunity. *Cancer Immunol Res*. 2018;6(1):14–24.
50. Theivanthiran B, Evans KS, DeVito NC, Plebanek MP, Sturdivant M, Wachsmuth LP, et al. A tumor-intrinsic PD-L1-NLRP3 inflammasome signaling pathway drives resistance to anti-PD-1 immunotherapy. *J Clin Invest*. 2020:133055.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

