Clinical and Translational Oncology (2021) 23:1743–1751
https://doi.org/10.1007/s12094-021-02575-z

RESEARCH ARTICLE

CDK1 promotes the stemness of lung cancer cells through interacting with Sox2

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Received: 8 January 2021 / Accepted: 15 February 2021 / Published online: 15 March 2021

Abstract

Objectives The promoting roles of cyclin dependent kinase 1 (CDK1) have been revealed in various tumors, however, its effects in the progression of cancer stem cells are still confusing. This work aims to explore the roles of CDK1 in regulating the stemness of lung cancer cells.

Methods Online dataset analysis was performed to evaluate the correlation between CDK1 exression and the survival of lung cancer patients. RT-qPCR, western blot, cell viability, sphere-formation analysis and ALDH activity detection were used to investigate the roles of CDK1 on lung cancer cell stemness, viability and chemotherapeutic sensitivity. Immunoco-precipitation (Co-IP) analysis and rescuing experiments were performed to reveal the underlying mechanisms contributing to CDK1-mediated effects on lung cancer cell stemness.

Results CDK1 mRNA expression was negatively correlated with the overall survival of lung cancer patients and remarkably increased in tumor spheres formed by lung cancer cells compared to the parental cells. Additionally, CDK1 positively regulated the stemness of lung cancer cells. Mechanistically, CDK1 could interact with Sox2 protein, but not other stemness markers (Oct4, Nanog and CD133). Furthermore, CDK1 increased the phosphorylation, cytoplasm-nuclear translocation and transcriptional activity of Sox2 protein in lung cancer cells. Moreover, CDK1 positively regulated the stemness of lung cancer cells in a Sox2-dependent manner. Finally, we revealed that inhibition of CDK1 enhanced the chemotherapeutic sensitivity, which was also rescued by Sox2 overexpression.

Conclusions This work reveals a novel CDK1/Sox2 axis responsible for maintaining the stemness of lung cancer cells.

Keywords CDK1 · Sox2 · Lung cancer · Cancer stem cell · Stemness

Introduction

Cancer stem cells (CSCs) have been identified in various tumors, including lung cancer [1]. Importantly, CSCs are regarded as the root of chemotherapeutic resistance and tumor progression [2]. Thus, it is very critical to elucidate the underlying mechanisms contributing to lung CSC progression.

Cell cycle is an orderly event in which a cell replicates its genome, grows and divides [3]. Cell cycle is regulated by many proteins, including cyclin, cyclin kinase, and cyclin kinase inhibitor. The occurrence and development of tumor cause cell cycle disorder by regulating some cell cycle factors. Cyclin-dependent kinase 1 (CDK1) plays an important role in cell cycle regulation, especially in mitosis [4]. Neganova et al. [5] found that CDK1 plays a role in regulating mitosis, G2/M checkpoint maintenance, cell apoptosis, cell pluripotency, and genome stability maintenance in multiple events of human pluripotent stem cells. Recently, CDK1-mediated phosphorylation of TFCP2L1 has been shown to be required for stem cell pluripotency and bladder carcinogeneis [6]. Additionally, CDK1 has been confirmed to be essential for the self-renewal of mouse embryonic stem cells [7]. As the normal stem cells and CSCs have similar development program, there is reason to believe that CDK1 is involved in CSC progression. As the previous study indicated that CDK1 promotes the tumor initiating ability of multiple cancer types [8, 9], which is positively correlated with the stemness of cancer cells. Additionally, CDK1 can regulate embryonic and induced pluripotency through

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activating the PDK1-mediated PI3K/Akt signaling pathway, which was recently shown to be responsible for modulating the cell cycle of CSC [10–12]. Additionally, a recent work has indicated that CDK1 serves as a potential prognostic biomarker for lung cancer [13]. Although the effects of CDK1 on lung cancer progression have been demonstrated and suppressing CDK1-related signaling can be targeted to inhibit lung cancer progression; however, it is still unclear whether CDK1 can regulate the stemness of lung cancer cells.

Currently, we showed that CDK1 is highly expressed in tumor spheres compared to the parental lung cancer cells, and negatively correlated with overall survival of lung cancer patients. Further functional experiments revealed that CDK1 positively regulated the stemness of lung cancer cells. Additionally, the expression of CDK1 and stemness markers (Oct4, Nanog, CD133) exhibited a positive correlation in lung cancer tissues. Mechanistic studies indicated that CDK1 interacted with Sox2, increased the phosphorylation level, nuclear localization, and transcriptional activity of Sox2, this is responsible for CDK1-mediated effects on lung cancer cell stemness. Notably, CDK1-induced reduction of chemotherapeutic sensitivity was rescued by Sox2 knockdown.

Material and methods

Cell culture

Lung adenocarcinoma cell line A549 and lung squamous cell carcinoma cell line NCI-H520 were purchased from ATCC (Manassas, VA, USA) and cultured in RPMI1640 medium (Hyclone, South Logan, UT, USA) with 10% fetal bovine serum (FBS, Hyclone) at 37 ℃.

Online dataset analysis

To analyze the correlation between transcript expression and the overall survival of lung adenocarcinoma and squamous cell carcinoma, online Kaplan–Meier (KM) Plotter analysis [14] was used, in which lung adenocarcinoma and squamous cell carcinoma patients were chosen, respectively. The other settings are the default settings. To evaluate the correlation between CDK1 expression and other transcript expression, the online R2: Genomics Analysis and Visualization Platform (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi) was used, and the lung adenocarcinoma and squamous cell carcinoma tissues were chosen, respectively.

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using RNA isolater Total RNA Extraction Reagent (Vazyme, Nanjing, China) and purified using RNeasy Maxi kit (Beyotime, Beijing, China). The A260/280 value of RNA was determined on nanodrop 2000c and values with 1.8–2.1 were used for further study. 1 μg total RNA was reverse transcribed into cDNA according to the operation instructions of HiScript II One Step RT-PCR Kit (Dye Plus) (Vazyme). Roche light-cycle 480 real-time PCR system was used to examine the mRNA levels of transcripts. GAPDH was used as the internal reference, and the relative expression levels of transcripts were calculated using the formula folds = $2^{-\Delta\Delta CT}$.

Plasmid construction and transfection analysis

The sequences of CDK1 were inserted into pcDNA4.1 vector, named as pc-CDK1. The siRNA sequences against Sox2 (si-Sox2) or CDK1 (si-CDK1) and corresponding negative control (NC) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The transfection procedure was performed using Lipofectamine 3000 (Thermo Fisher Scientific,Waltham, MA, USA) following the manusfacturer's recommendation.

Western blot

The detailed procedure was referred to the previous study [15]. After exposed with High sensitive ECL chemiluminescence detection kit (Vazyme), the gray value of the bands was read automatically by the gel imager (Bio-Rad, Hercules, CA, USA). β-actin was used as the internal reference.

Sphere-formation analysis

To analyze the sphere-formation ability of lung cancer cells with different treatments, 20 ng/ml epidermal growth factor (EGF, PeproTech), 20 ng/ml basic fibroblast growth factor (bFGF, PeproTech, Rocky Hill, NJ, USA), 0.4% bovine serum albumin (BSA, Invitrogen, Carlsbad, CA, USA), 5 ng/ml insulin (Sigma-Aldrich, St. Louis, MO, USA) and 0.02 × B27 were added into serum-free DMEM/F12 medium (Hyclone) to culture cells at ultra-low adhesion 6-well plates with a 1000 cells/ml density. The culture medium was supplemented every 3 days. 10 days later, spheres more than 50 μm were calculated and photographed.

Immunocoprecipitation (Co-IP) analysis

Briefly, cells were washed with pre-cooled PBS and lysed with the pre-cooled RIPA buffer. Cell suspension was centrifuged for 20 min at 4 ℃ by 13,000 rpm, and collect the supernatant. The detailed procedure was referred to

the previous study using Protein A agarose beads (50%) (Bimike, Shanghai, China) [16].

ALDH activity detection

The ALDH Activity Assay Kit (Abcam, Cambridge, MA, UK) was used to analyze ALDH activity following the manufacturer's recommendation.

Cell viability assay

Cells were seeded in 96-well plates at 3000 cells/well density. After 12 h, cells were treated with cisplatin as well as CDK1 overexpression and sox2 knockdown or not for another 24, 48 h and 72, respectively. Then WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-isulfophenyl)-2H-tetrazolium, monosodium salt] was added into wells according to the protocol mentioned in Cell Counting Kit-8 (CCK-8) kit (MedChem Express, Monmouth Junction, NJ,USA), and incubated for 2 h. Then, the absorbance at 450 nm was measured using a microplate reader.

Statistical analysis

All data were denoted as the mean ± SD. Prism7 (GraphPad software, Version X; La Jolla, CA, USA) was used for statistical analysis. The significance was analyzed using the student's *t*-test and one-way ANOVA with the Tukey–Kramer post-test. P value < 0.05 or less was considered significant.

Results

CDK1 expression is positively correlated with the expression of stemness markers in lung cancer tissues

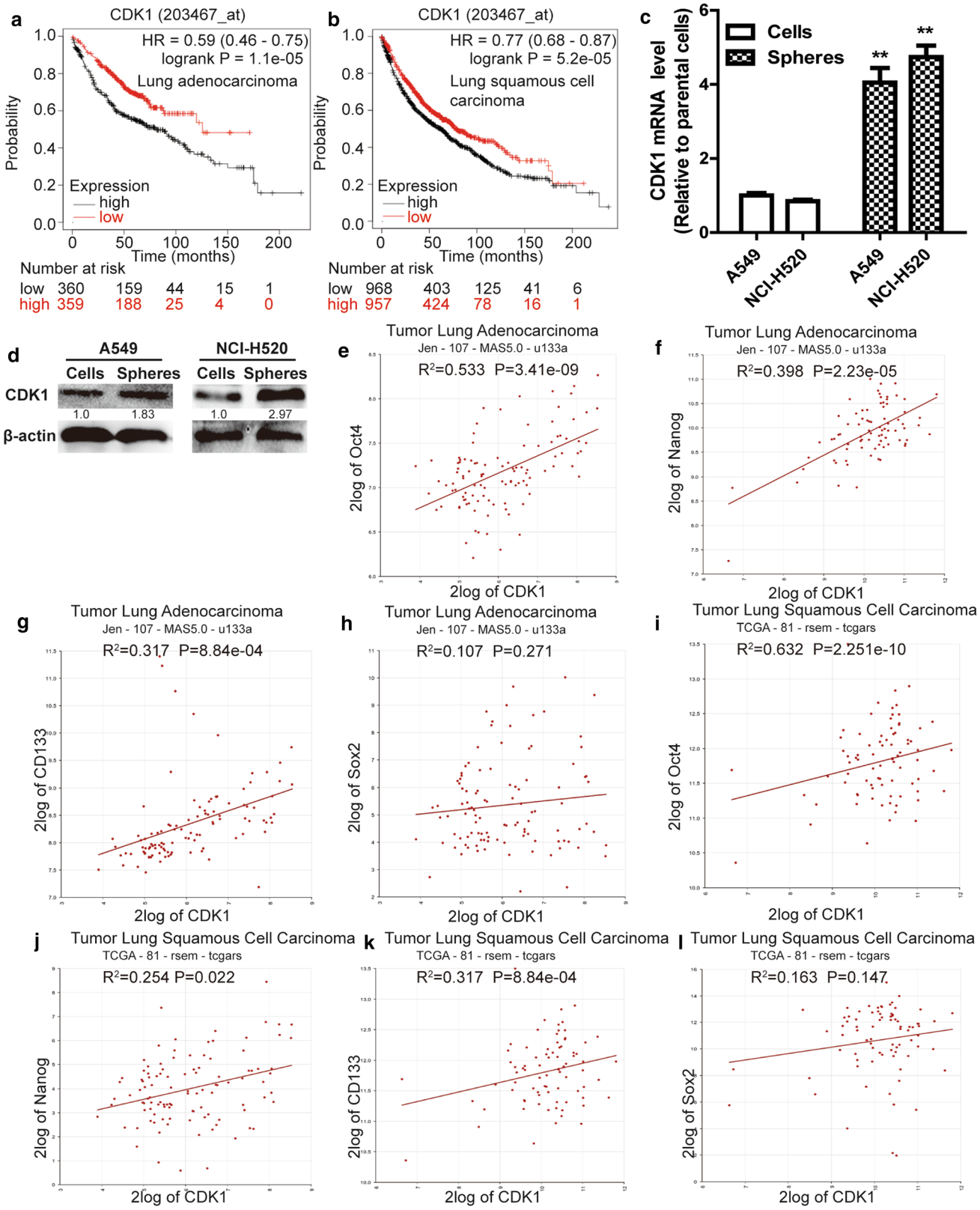
We initially found that CDK1 mRNA expression was negatively correlated with the overall survival of lung adenocarcinoma (Fig. 1a) and squamous cell carcinoma (Fig. 1b) patients through the online Kaplan—Meier (KM) Plotter analysis [14]. To explore whether CDK1 can regulate the stemness of lung cancer cells, we detected the expression of CDK1 in lung cancer spheres and cells, and found that CDK1 expression was remarkably increased in tumor spheres compared with the parental cells (Fig. 1c, d). Notably, CDK1 mRNA expression is positively correlated with the expression of stemness markers (Oct4, Nanog, CD133) in lung adenocarcinoma and squamous cell carcinoma using the online R2: Genomics Analysis and Visualization Platform (Fig. 1e–k). However, there is no correlation between CDK1 and Sox2 mRNA expression, which is another critical stemness regulator (Fig. 1h, l). These results suggest that CDK1 may regulate the stemness of lung cancer cells.

CDK1 positively regulates the stemness of lung cancer cells

To confirm the effects of CDK1 on the stemness of lung cancer cells, CDK1 was overexpressed or knocked down in lung cancer cells. The overexpression and knockdown efficiency was proved by Western blot analysis (Fig. 2a). As shown in Fig. 2b, c, overexpression of CDK1 increased the sphere-formation ability, while CDK1 knockdown exerted an opposite effect, which was characterized by the change of sphere size and number. Additionally, the expression of stemness markers (Oct4, Nanog, CD133), but not Sox2, was increased in lung cancer cells with CDK1 overexpression and decreased in cells with CDK1 knockdown, respectively (Fig. 2d). Furthermore, ALDH activity was increased or decreased in lung cancer cells with CDK1 overexpression or knockdown, respectively (Fig. 2e). Notably, we performed overexpression experiments with pc-CDK1 in absence and presence of CDK1 specific inhibitor (Ro-3306) to observe the change of lung cancer cell stemness. As shown in Fig. 3, Ro-3306 suppressed the promoting effects of pc-CDK1 on the stemness of lung cancer cells. These results demonstrate that CDK1 can promote the stemness of lung cancer cells.

CDK1 directly interacts with Sox2 protein, increases Sox2 phosphorylation and thus enhances Sox2 transcriptional activity

Since CDK1 belongs to a protein kinase, we wonder whether CDK1 can directly interact with these stemness markers (Oct4, Nanog, CD133). However, the co-IP results showed that CDK1 did not interact with Oct4, Nanog and CD133 (Fig. 4a). As the previous study showed that CDK1 can interact with Sox2 in human melanoma and the stemness markers (Oct4, Nanog) act as the downstream effectors of Sox2, we speculate that the CDK1-Sox2 interaction exists in lung cancer too. As expected, CDK1 interacted with Sox2 reciprocally in lung cancer cells (Fig. 4a, b). As CDK1 cannot change Sox2 expression, we wondered whether CDK1 can regulate the phosphorylation and thus the transcriptional activity of Sox2. The expression of the target genes (UTF1, FGF4) was detected in lung cancer cells with ectopic expression of Sox2 and we found that CDK1 indeed positively regulated the expression of UTF1 and FGF4 (Fig. 4c–e). In consistent, CDK1 overexpression led to an increase in Sox2 phosphorylation, while CDK1 knockdown exerted an opposite effect in lung cancer cells (Fig. 4f). Additionally, CDK1 overexpression promoted the nuclear localization of Sox2 (Fig. 4g).


a
CDK1 (203467_at)
HR = 0.59 (0.46 - 0.75)
logrank P = 1.1e-05
Lung adenocarcinoma
Probability
Expression
high
low
Time (months)
Number at risk
low 360 159 44 15 1
high 359 188 25 4 0
b
CDK1 (203467_at)
HR = 0.77 (0.68 - 0.87)
logrank P = 5.2e-05
Lung squamous cell carcinoma
Probability
Expression
high
low
Time (months)
Number at risk
low 968 403 125 41 6
high 957 424 78 16 1
c
CDK1 mRNA level (Relative to parental cells)
Cells
Spheres
**
**
A549
NCI-H520
A549
NCI-H520
d
A549
NCI-H520
Cells Spheres Cells Spheres
CDK1
1.0 1.83 1.0 2.97
β-actin
e
Tumor Lung Adenocarcinoma
Jen - 107 - MAS5.0 - u133a
R²=0.533 P=3.41e-09
2log of Oct4
2log of CDK1
f
Tumor Lung Adenocarcinoma
Jen - 107 - MAS5.0 - u133a
R²=0.398 P=2.23e-05
2log of Nanog
2log of CDK1
g
Tumor Lung Adenocarcinoma
Jen - 107 - MAS5.0 - u133a
R²=0.317 P=8.84e-04
2log of CD133
2log of CDK1
h
Tumor Lung Adenocarcinoma
Jen - 107 - MAS5.0 - u133a
R²=0.107 P=0.271
2log of Sox2
2log of CDK1
i
Tumor Lung Squamous Cell Carcinoma
TCGA - 81 - rsem - tcgars
R²=0.632 P=2.251e-10
2log of Oct4
2log of CDK1
j
Tumor Lung Squamous Cell Carcinoma
TCGA - 81 - rsem - tcgars
R²=0.254 P=0.022
2log of Nanog
2log of CDK1
k
Tumor Lung Squamous Cell Carcinoma
TCGA - 81 - rsem - tcgars
R²=0.317 P=8.84e-04
2log of CD133
2log of CDK1
l
Tumor Lung Squamous Cell Carcinoma
TCGA - 81 - rsem - tcgars
R²=0.163 P=0.147
2log of Sox2
2log of CDK1

◂**Fig. 1** CDK1 expression is positively correlated with the expression of stemness markers in lung cancer tissues. **a**, **b** The correlation between CDK1 mRNA expression and overall survival of lung adenocarcinoma and squamous cell carcinoma patients was examined via the online Kaplan–Meier (KM) Plotter analysis. **c**, **d** The mRNA (**c**) and protein (**d**) levels of CDK1 were detected in lung cancer cells and spheres. Data are presented as the mean ± SD, $n \geq 3$, $^{**}P < 0.01$ vs. cells. **e**–**h** The correlation between CDK1 and stemness markers expression was determined in lung adenocarcinoma tissues via online R2: Genomics Analysis and Visualization Platform. **i**–**l** The correlation between CDK1 and stemness markers expression was determined in lung squamous cell carcinoma tissues via online R2: Genomics Analysis and Visualization Platform

CDK1 promotes the stemness of lung cancer cells dependent on Sox2

Finally, we investigated the necessity of Sox2 for CDK1-mediated effects on the stemness of lung cancer cells. Sox2 was knocked down in lung cancer cells with CDK1 overexpression and the co-transfection efficiency was confirmed (Fig. 5a). As shown in Fig. 5b, c, Sox2 knockdown rescued the promoting effects of CDK1 overexpression on the sphere-formation ability. Additionally, CDK1-induced upregulation of stemness marker expression was attenuated by Sox2 knockdown (Fig. 5d). Furthermore, the enhanced ALDH activity led by CDK1 overexpression was partially abrogated by Sox2 knockdown (Fig. 5e). Moreover, CDK1 overexpression attenuated the chemotherapeutic sensitivity of lung cancer cells, which was rescued by Sox2 knockdown (Fig. 5f, g). Therefore, our results suggest that CDK1 positively regulates the stemness of lung cancer cells dependent on Sox2.

Discussion

Targeting CSCs have been regarded as a novel strategy for effectively eradicating cancer [17]. Indeed, many drugs are being explored to target CSCs [18]; however, there is still no drugs targeting CSCs in clinical, this might be due to the unclear mechanisms contributing CSC progression.

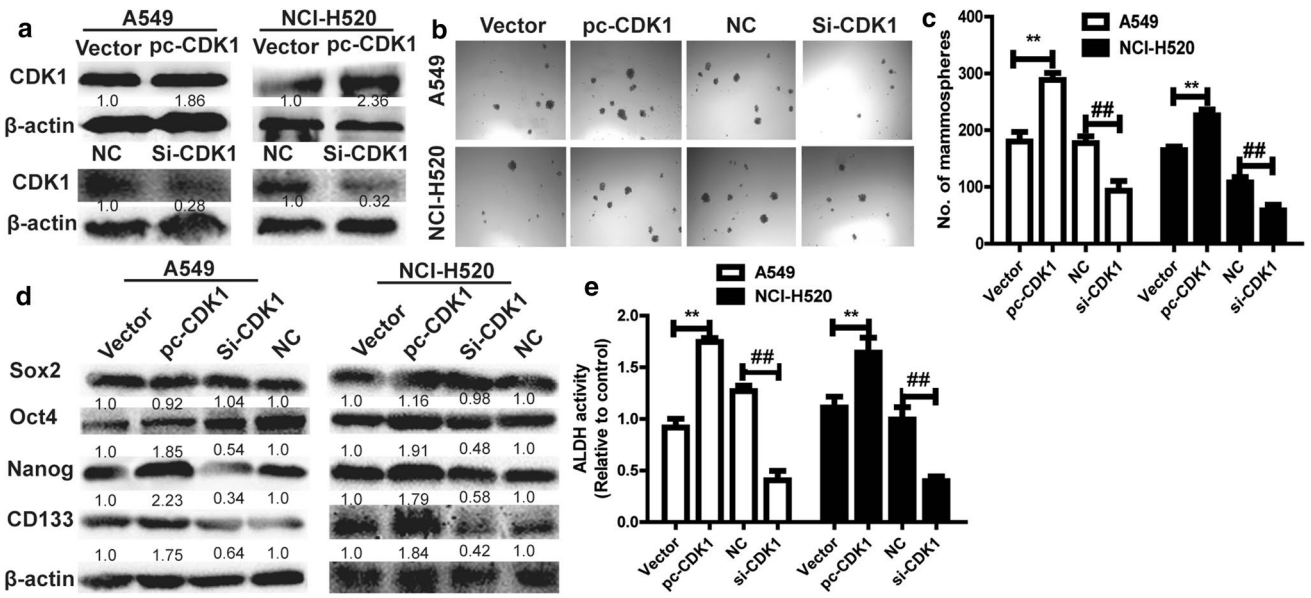


Fig. 2 CDK1 positively regulates the stemness of lung cancer cells. **a** CDK1 protein expression was detected in lung cancer cells with CDK1 overexpression or knockdown. **b** Sphere size was determined in lung cancer cells with CDK1 overexpression or knockdown. **c** Sphere number was counted in lung cancer cells with CDK1 overexpression or knockdown. **d** The expression of stemness markers (Sox2, Oct4, Nanog, CD133) was examined in lung cancer cells with CDK1 overexpression or knockdown. **e** ALDH activity was evaluated in lung cancer cells with CDK1 overexpression or knockdown. Data are presented as the mean ± SD, $n \geq 3$, $^{**}P < 0.01$ vs. vector, $^{\#\#}P < 0.01$ vs. NC

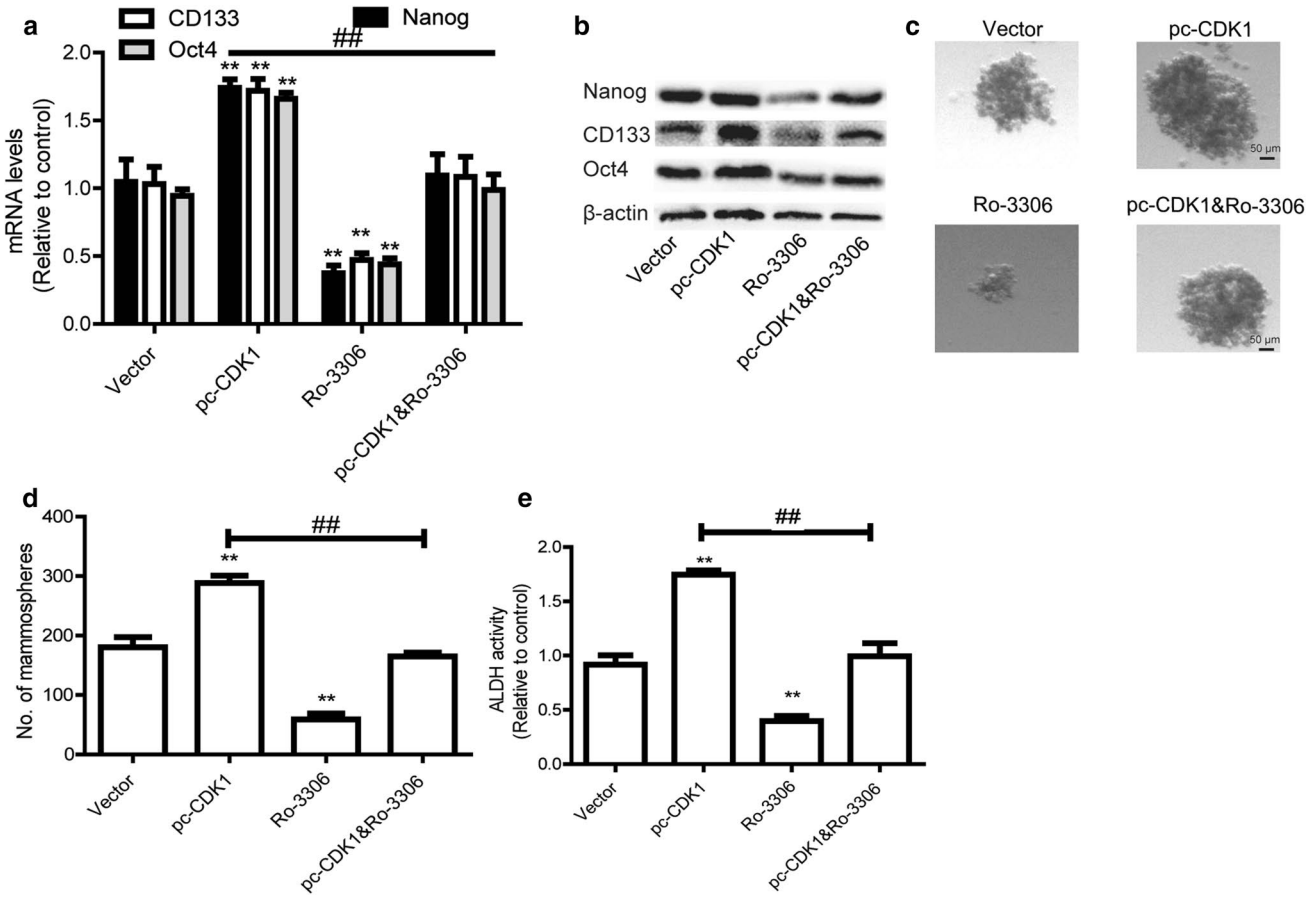


Fig. 3 CDK1 inhibition suppressed the promoting effects of CDK1 overexpression on the stemness of A549 cells. **a** The mRNA expression of stemness markers (Oct4, Nanog, CD133) was examined in A549 cells with CDK1 overexpression as well as Ro-3306 treatment or not. **b** The protein expression of stemness markers (Oct4, Nanog, CD133) was examined in A549 cells with CDK1 overexpression as well as Ro-3306 treatment or not. **c** Sphere size was determined in A549 cells described in (**a**). **d** Sphere number was counted in A549 cells depicted in (**a**). **e** ALDH activity was evaluated in A549 cells described in (**a**). Data are presented as the mean ± SD, $n \geq 3$, $^{**}P < 0.01$ vs. vector, $^{\#\#}P < 0.01$ vs. pc-CDK1

CSCs are usually dormant and inactive in proliferation [2]. Most of the chemotherapeutic drugs are mainly aimed at splitting tumor cells, which are usually in G2/S phase and CSCs in G0/G1 phase are not sensitive to chemotherapeutic drugs [19]. Radiotherapy and chemotherapy eliminated common tumor cells and enriched CSCs. Meanwhile, due to the stimulation of chemotherapeutic drugs, static CSCs quickly wake up, enter the cell cycle, rapidly proliferate and divide, reconstruct the whole tumor phenotype, leading to tumor recurrence. Therefore, regulating the cell cycle might be a potential strategy for killing CSCs. In the present study, we revealed that CDK1 positively regulated the stemenss of lung cancer cells including lung adenocarcinoma and squamous cell carcinoma cells, which supported by the following results: first, CDK1 positively regulates the expression of stemness makers; second, CDK1 positively regulates the ability of sphere-formation; third, CDK1 positively regulates the activity of ALDH.

CDK1 has been found to be a critical regulator for cell cycle and it is essential for muscle regeneration and overload muscle fiber hypertrophy [20], which is similar with the CSC self-renewal ability. Additionally, CDK1 activity is tightly correlated with the instability of chromosomal [21], which has been confirmed to be resulted by the existence of CSC. There are many substrates or partners of CDK1 have been identified, such as N-methyl-D-aspartate receptors (NMDAR) [22], ICP18 [23] and Dam1 complex [24]. Here, we found that CDK1 can directly interact with Sox2, a key stemness regulator, which is consistent with the previous study [8]. We wonder whether

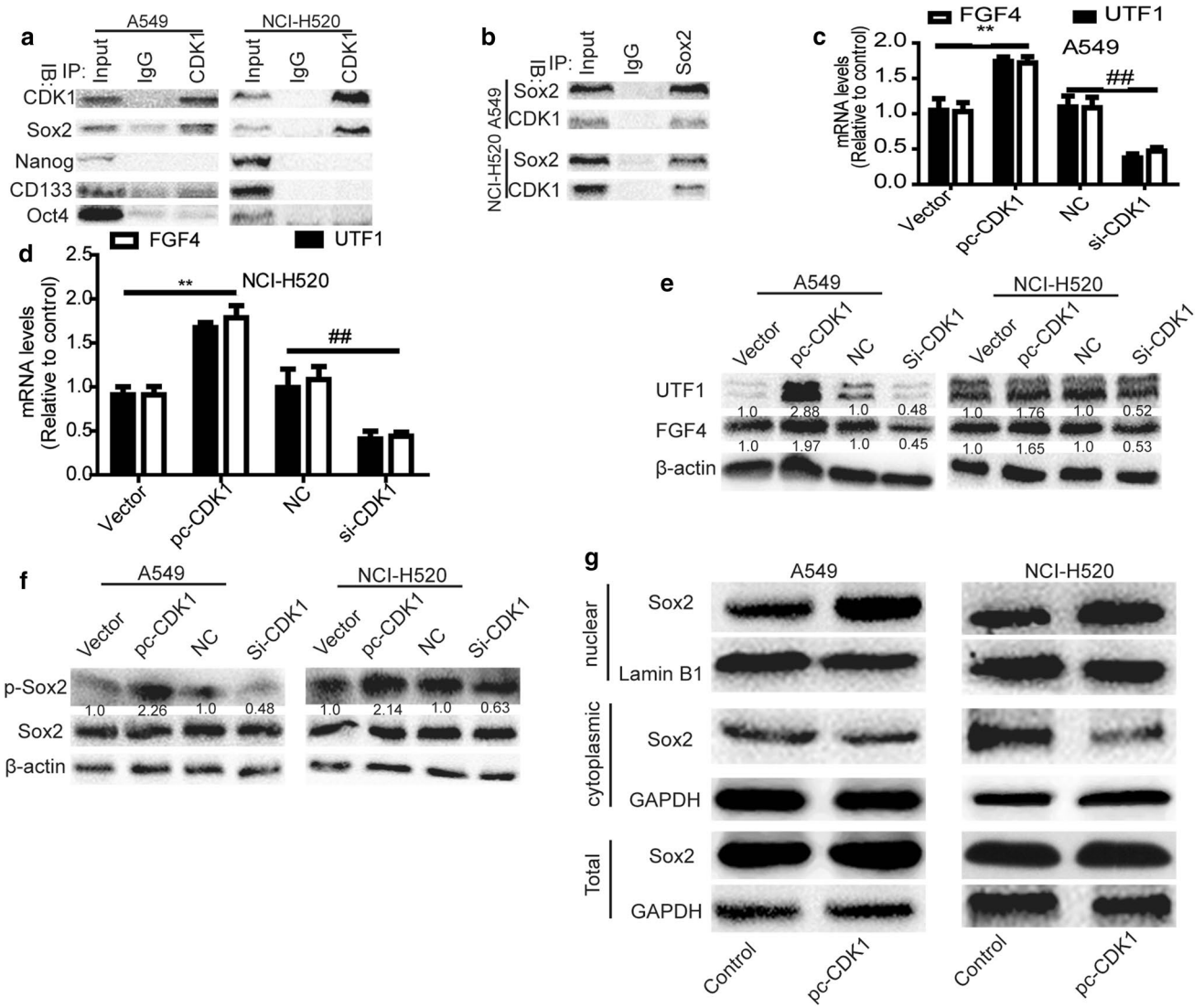


Fig. 4 CDK1 directly interacts with Sox2 protein, increases Sox2 phosphorylation and thus enhances Sox2 transcriptional activity. **a**, **b** The interaction between CDK1 and stemness markers (Sox2, Oct4, Nanog, CD133) was detected in lung cancer cells via co-IP experiments. **c**, **d** The mRNA levels of FGF4 and UTF1 were examined in lung cancer cells with CDK1 overexpression or knockdown. Data are presented as the mean ± SD, $n \geq 3$, $^{**}P < 0.01$ vs. vector, $^{\#\#}P < 0.01$ vs. NC. **e** The protein level of FGF4 and UTF1 was determined in lung cancer cells with CDK1 overexpression or knockdown. **f** The expression of p-Sox2 was evaluated in lung cancer cells with CDK1 overexpression or knockdown. **g** The nuclear and cytoplasmic sox2 protein expression was detected in lung cancer cells with or without CDK1 overexpression

this CDK1/Sox2 axis is a common phenomenon in other types of tumor. However, it is still unclear whether the other partners are involved in CDK1-mediated effects on lung cancer cell stemness. Furthermore, our results showed that CDK1 can enhance the phosphorylation, cytoplasm-nuclear translocation, and the transcriptional activity of Sox2. However, a previous work demonstrates that CDK1 interplays with Oct4, another stemness regulator, to suppress the differentiation of embryonic stem cells into trophectoderm [25], this is inconsistent with our results showing that CDK1 cannot interact with Oct4 in lung cancer cells. We speculate that CDK1 have different substrates or partners in different type of cells, especially distinguishing in normal and cancer cells. Notably, since there are several inhibitors of CDK1 that have been established, such as K00546, NU6027, Ro-3306, and BMS-265246, targeting CDK1 might be explored to kill lung CSCs although the specificity of these CDK1 should be improved in future. Importantly, as our results demonstrate that overexpression of CDK1 attenuate the chemosensitivity in lung cancer cells and inhibition of CDK1 has been confirmed to reverse the resistance 5-Fu in colorectal

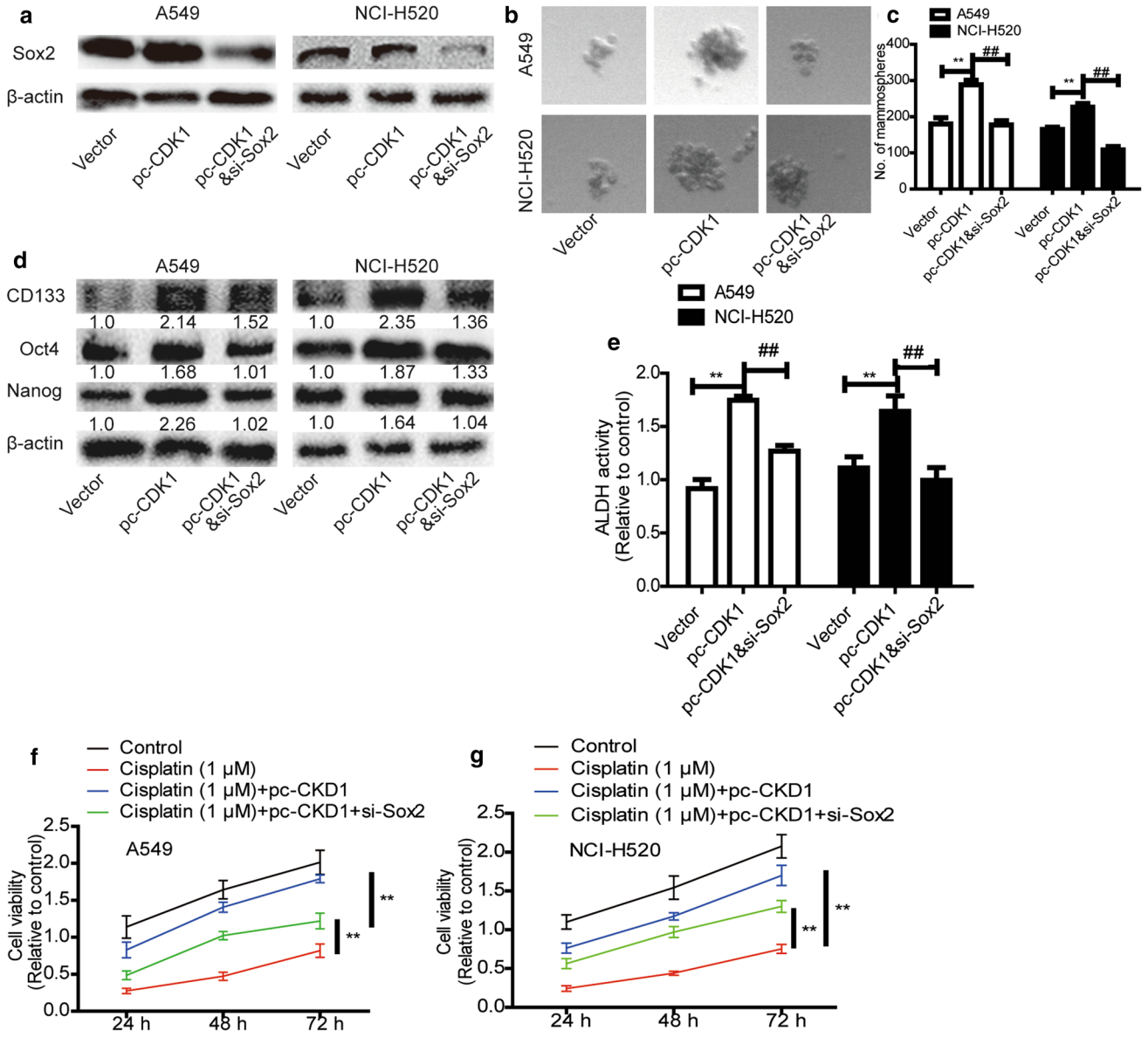


Fig. 5 CDK1 promotes the stemness of lung cancer cells dependent on Sox2. a Sox2 protein expression was detected in lung cancer cells with CDK1 overexpression as well as Sox2 knockdown or not. **b**, **c** Sphere-formation ability was evaluated in lung cancer cells with CDK1 overexpression as well as Sox2 knockdown or not. Data are presented as the mean ± SD, $n \geq 3$, $^{**}P < 0.01$ vs. vector, $^{\#\#}P < 0.01$ vs. pc-CDK1. **d** The protein expression of stemness markers was examined in lung cancer cells with CDK1 overexpression plus Sox2 knockdown or not. **e** ALDH activity was determined in lung cancer cells with CDK1 overexpression plus Sox2 knockdown or not. Data are presented as the mean ± SD, $n \geq 3$, $^{**}P < 0.01$ vs. vector, $^{\#\#}P < 0.01$ vs. pc-CDK1. **f**, **g** The cisplatin sensitivity was evaluated in lung cancer cells with CDK1 overexpression plus Sox2 knockdown or not. Data are presented as the mean ± SD, $n \geq 3$, $^{**}P < 0.01$ vs. cisplatin (1 μM)

cancer cells, [26] CDK1 inhibitor might be combined with chemotherapeutic drugs in clinical for lung cancer patients. We must admit that further in vivo experiments should be performed to prove the effects of CDK1 on the stemness of lung cancer cells.

Taken together, this study first reveals that CDK1 can promote the stemness of lung cancer cells through interplaying with Sox2 and thus enhancing Sox2 transcriptional activity. This CDK1/Sox2 axis might be targeted to treat lung cancer, especially chemoresistant lung cancer.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

Informed consent For this type of study, formal consent is not required.

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