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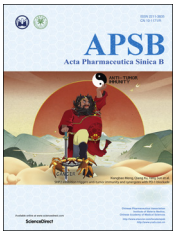

ORIGINAL ARTICLE

LSD1 inhibition suppresses the growth of clear cell renal cell carcinoma *via* upregulating P21 signaling

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Abstract Histone lysine-specific demethylase 1 (LSD1) has been implicated in the disease progression of several types of solid tumors. This study provides the first evidence showing that LSD1 overexpression occurred in 62.6% (224/358) of clear cell renal cell carcinomas (ccRCC). LSD1 expression was associated with the progression of ccRCC, as indicated by TNM stage ($P=0.006$), especially tumor stage ($P=0.017$) and lymph node metastasis ($P=0.030$). High LSD1 expression proved to be an independent prognostic factor for poor overall survival ($P<0.001$) and recurrence-free survival ($P<0.001$) of ccRCC patients. We further show that LSD1 inhibition by siRNA knockdown or using the small molecule inhibitor SP2509 suppressed the growth of ccRCC *in vitro* and *in vivo*. Mechanistically, inhibition of LSD1 decreased the H3K4 demethylation at the *CDKN1A* gene promoter, which was associated with P21 upregulation and cell cycle arrest at G1/S in ccRCC cells. Our findings provide new mechanistic insights into the role of LSD1 in ccRCC and suggest the therapeutic potential of LSD1 inhibitors in ccRCC treatment.

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1. Introduction

Kidney cancer is the most common lethal urological malignancy[1]. The incidence of kidney cancer has been increasing in recent decades, causing 134,000 deaths annually worldwide[2]. Renal cell carcinoma (RCC) is the major type of kidney cancer, among which the clear cell renal cell carcinoma (ccRCC) is the most common pathological subtype (65%–70%)[3]. Nearly one third of patients are diagnosed with advanced stage disease, and almost 20% of localized RCC patients progressed to local recurrence and distant metastasis, even after radical surgeries[2]. Inactivation of the von Hippel-Lindau (*VHL*) gene by gene mutation, deletion or epigenetic dysregulation causes overexpression of hypoxia-inducible factor (HIF) pathway components, and is considered one of the most crucial carcinogenic factors for ccRCC[4]. Currently, anti-angiogenic therapies such as the tyrosine kinase inhibitors sunitinib, sorafenib and the mammalian target of rapamycin (mTOR) inhibitor everolimus, which may activate upstream phosphatidylinositol 3-kinase (PI3K)[5], have been approved as the standard of care for metastatic ccRCC[6] and have significantly improved the survival of ccRCC patients. However, few patients achieve complete remission and a significant number of responsive patients develop resistance over time. Although there have been several studies to find biomarkers in RCC patients[7,8], it remains imperative to identify targets for new therapeutic approaches[9].

Deregulation of epigenetic regulators has been implicated in cancer progression and the development of drug resistance in multiple cancers including RCC[10]. Histone lysine methylation is known as an important histone modification and plays a pivotal role in embryonic development and tumorigenesis[11–14]. LSD1 (also known as KDM1A) is the first identified histone lysine-specific demethylase, which catalyzes the demethylation of di- and mono-methylated histone H3 lysine 4 (H3K4) or lysine 9 (H3K9) residues[15]. LSD1 acts as a transcriptional co-repressor that participates in transcriptional regulation of the formation of complexes like RESE/Co-REST and is associated with various cellular processes[16,17]. The precise molecular mechanism of LSD1 in transcriptional regulation has not been elucidated. Increasing evidence has suggested an association between high LSD1 expression and poor prognosis in some human malignancies, such as leukemia[18] and some solid tumors[19–22]. We also noticed that LSD1 was implicated in maintaining the HIF-1α level *via* demethylation under hypoxic conditions[23]. Given the profound involvement of the HIF signaling pathway in RCC progression[24], we asked whether LSD1 plays an important role in RCC development and may serve as a target for therapeutic intervention.

In this study, we investigated LSD1 expression in human ccRCC samples and examined its association with clinical progression of ccRCC. We also examined the antineoplastic activity of LSD1 inhibitors in ccRCC cell lines and xenograft models, and further explored the mechanism by which LSD inhibitors induce suppression of ccRCC cell lines.

2. Materials and methods

2.1. Tissue samples and immunohistochemistry

Tissue microarrays (TMAs) were obtained from 358 ccRCC patients who underwent nephrectomy surgery in Renji Hospital, School of Medicine, Shanghai Jiaotong University. TMAs were made using these tissues in Shanghai Outdo Biotech Company (Shanghai, China) including tumor and adjacent tissues. Immunohistochemical (IHC) analysis of LSD1 protein levels was performed according to the standard streptavidin–peroxidase method (Zymed Laboratories Inc, San Francisco, CA, USA). The primary antibody against LSD1 (Anti-KDM1/LSD1, Abcam, Cambridge, MA, USA) was diluted 1:50. PBS instead of primary antibody served as the negative control. Immunostaining of LSD1 protein was examined and assessed independently by two observers, and calculated as the intensity of the staining and as a cell percentage. The final staining score was divided according the percentage of positive cells: 1 (0–25%), 2 (26%–50%), 3 (51%–75%), and 4 (>75%), also the intensity of staining was classified as 0 (negative), 1 (weak), 2 (moderate), 3 (strong) (Supporting Information Fig. S1). The total IHC score was calculate by staining percentage × intensity. The expression of LSD1 was divided into two groups: low expression was indicated by a score < 6, while high expression indicated a score ≥ 6, as in a previous study. Twenty fresh and frozen tissue samples were collected from 10 ccRCC patients for Western blot and quantitative real-time PCR (QRT-PCR) analysis immediately after radical nephrectomy surgery. Written informed consent was obtained from all patients.

2.2. Western blot analysis

Western blotting was performed according to the standard protocol with the protein lysates harvested form fresh tumor samples and cultured cells. Two μg of total protein was applied to one end of a 12% SDS polyacrylamide gel. After electrophoresis proteins on the gel were transferred onto a nitrocellulose membrane (Millipore, Temecula, CA, USA). After blocking with non-fat milk for almost 1 h at room temperature, the membranes were incubated overnight at 4 °C with primary antibodies: anti-LSD1 (1:1000), anti-H3K4me2 (1:1000), anti-H3K9me2 (1:1000), anti-P53(1:500), anti-P21 (1:500), anti-CDK4 (1:1000), anti-CDK6 (1:1000), anti-GAPDH (1:2000) and anti-β-actin (1:1000), which were all purchased from Cell Signaling Technology (Boston, Massachusetts, USA). Membranes were washed three times and incubated with secondary antibodies (1:500; Abcam, Cambridge, MA, USA) at room temperature for 1 h. Immunoreactive bands were detected by using Amersham Hyper lm ECL (GE Healthcare Life Sciences). GAPDH and β-actin were used as loading controls.

2.3. Cell culture and SP2509 preparation

The renal tumor cell lines of 786-O, CAKI-1, A498, 769-P, ACHN and the normal cell line HK-2 were supplied by the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were maintained in the recommended medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Australia), 1% GlutaMAX, 1% nonessential amino acids, and 1% sodium pyruvate. Cultures were maintained at 37 °C with 5% CO_2 and the medium was changed at least once weekly. Cells with fewer than 50 passages were used for experiments.

SP2509 was provided by MedChemExpress (New Jersey, USA). The chemical reagent was diluted into dimethyl sulfoxide (DMSO) according to the instructions.

2.4. Gene silencing by siRNA transfection

786-O and CAKI-1 cell lines associated with high LSD1 expression were selected for further research and seeded in six-well

plates. The LSD1 and negative control small interfering RNAs (siRNA) (50–100 μmol/L) were transfected with lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The sequences are as follows: siLSD11, 5′-CUACAUCUUACCUUAGUCATT-3′ (sense), 5′-UGACUAAGGUAAGAUGUAGTT-3′ (antisense); siLSD12, 5′-CAGCUGACAUUUGAGGCUATT-3′ (sense), 5′-UAGCCUCAAAUGUCAGCUGTT-3′ (antisense), RNA negative control, 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense), 5′-ACGUGACACGUUCGGAGAATT-3′ (antisense). LSD1 expression levels were measured by Western blot analysis after 48 h.

2.5. Cell proliferation assay

786-O and CAKI-1 cells were plated into 96-well plates (2×10^3 cells/well) and incubated overnight at 37 °C. The siRNA targeting LSD1 and negative control were transduced into cells, then cell counts were taken every 24 h for 96 h post transfection. The effects of SP2509 on RCC cell survival were determined by sulforhodamine B (SRB) assay. After treatment, cells were incubated in cold TCA (10%) at 4 °C for 1 h and then washed and stained with 0.4% SRB into each well for 15–20 min at room temperature. After washing with 1% acetic acid, cell viability was assessed by measuring absorbance at 560 nm in 10 mmol/L Tris on a Soft Max pro plate reader. Drug interaction was estimated by an inhibition rate. All procedures were repeated three times or more.

2.6. Colony growth assay

Cultured RCC cell lines were seeded in 6-well plates at a density of 500 cells per well with low-dose SP2509 treatment. Colony formation was measured at day 7 after plating, and the colonies were stained with crystal violet, then photographed and counted.

2.7. Cell cycle assay

A cell cycle assay after LSD1 knock-down and drug treatment was performed as previous reported; firstly, cells were collected and fixed in 70% pre-chilled ethanol at 4 °C overnight and then stained with propidium iodide (PI) and analyzed by FACS flow cytometry (Becton-Dickinson, Mountain View, CA)[25].

2.8. Cell apoptotic analysis

A flow cytometer was used to identify cell apoptosis using the annexin V-FITC apoptosis detection kit (Becton-Dickinson, New Jersey, USA) according to the manufacturer's instructions. Data were analyzed with FlowJo software (FlowJo Version 10.0.7, USA).

2.9. Quantitative real-time PCR and chromatin immunoprecipitation analyses

QRT-PCR analysis was performed as previously described[25], and a human β-actin was used to normalized the measurement of cDNA between different samples. PCR primer sequences are listed in Supporting Information Table S1. Following 48 h interference, cell collection, cross-linking, cell lysis, sonication and chromatin immunoprecipitation for LSD1 and H3K4me2 were performed according to the manufacturer's protocol (SimpleChIP® Plus Enzymatic Chromatin IP Kit9005, Cell Signal Technology, Boston, Massachusetts, USA). Relative enrichment of the P21 promoter DNA in the chromatin immunoprecipitates was normalized against that in the input samples following the manufacturer's instructions.

2.10. RNA-sequence and data analysis

RNA-sequence (RNA-seq) analysis was performed as described previously[26]. CLC Genomics Workbench (Qiagen Bioinformatics) was used for analysis of the gene ontology according to a standard protocol.

2.11. In vivo model of RCC

All *in vivo* studies were approved by the Experimental Animal Ethics Committee of Shanghai JiaoTong University. Six-week-old female athymic mice were used in this study, and tumor xenografts were established by injection of 3×10^6 CAKI-1 and 786-O cells with 1:1 matrigel (BD, USA) into flank region of the mice. The following treatments were carried out in different groups of 6 mice for each RCC cell line: negative control (vehicle group) and 15 mg/kg SP2509 group. SP2509 (formulated with 10% DMSO, 30% Cremaphor, 60% sterile water) was administered daily intraperitoneally for 4 weeks. Mice were measured and checked twice a week; tumors were excised at the end of the *in vivo* experiments and the tumor samples collected and saved in 4% paraformaldehyde for further IHC staining analysis.

2.12. Statistical analysis

LSD1 expression and clinicopathologic characteristics were determined by using the χ^2 test or Fisher's exact test. Univariate and multivariate analysis was used to test for the independent prognosis. Overall survival and recurrence-free survival curves were plotted by the Kaplan-Meier method and compared with the log-rank test. Significant differences between values obtained from the cell lines treated with SP2509 and transfected with siRNA were determined using a two-tailed, paired *t*-test analysis using GraphPad Prism (GraphPad Software, Inc., CA, USA). All *P* values of less than 0.05 were assigned significance.

3. Results

3.1. Higher LSD1 expression is associated with poor prognosis in ccRCC patients

We firstly extracted the LSD1 expression in kidney cancer from the GENT (Gene Expression of Normal and Tumor Tissues) database (http://medicalgenome.kribb.re.kr/GENT/), which is a public database providing the gene expression patterns across diverse cancers and normal tissues[27]. We found that the LSD1 expression level was significantly higher in kidney cancer compared with normal tissues (Fig. 1A). This result was recapitulated in 10 pairs of ccRCC specimens and the corresponding normal tissues using the qPCR assay (Fig. 1B). We also discovered that the LSD1 protein level was upregulated in most ccRCC samples compared to normal kidney tissues (Fig. 1C and D).

We next investigated whether LSD1 expression might be associated with the prognosis of ccRCC patients. To this end, we examined the LSD1 expression in a tissue microarray consisting of 358 ccRCC samples using immunochemical (IHC) staining. The patients'

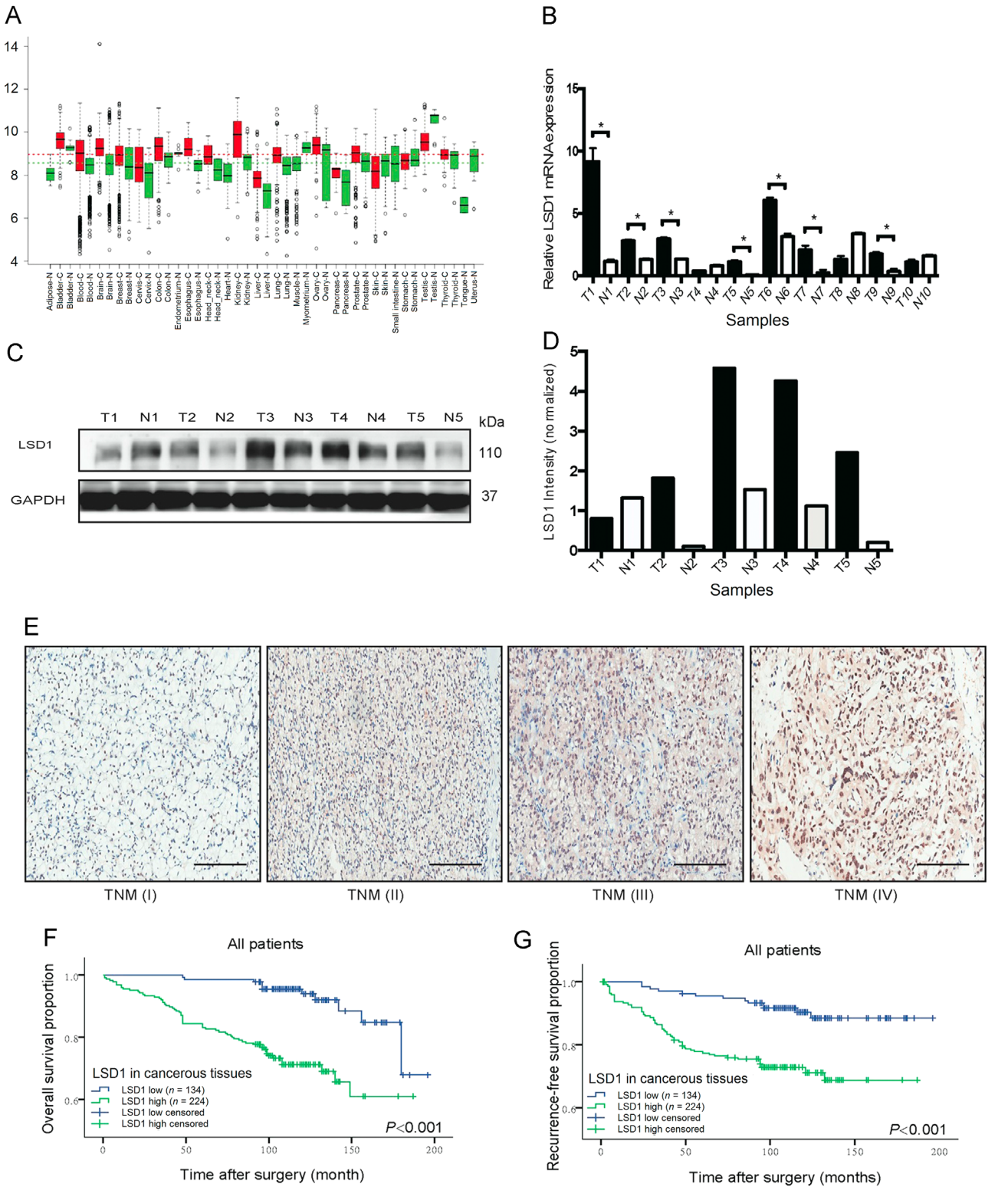

Figure 1 LSD1 is upregulated in RCC samples. A, GENT database of LSD1 among cancerous and normal kidney, showing high LSD1 expression in kidney cancer (red) (Download from GENT website). B, *LSD1* relative mRNA expression shown for RCC tissues and the matched normal samples in 10 patients (T means tumor and N means normal). C, Western blot analysis from RCC samples and distant-site normal samples with GAPDH as the loading control. D, Quantitation of LSD1 expression by protein content by Western blotting. E, The representative images of TNM by degree of ccRCC tissues, bar 100 μm. LSD1 expression was strongly correlated with TNM degree. F, The association between LSD1 expression level in tumors and OS in RCC patients. Kaplan-Meier and log-rank test analysis were used to compare the two groups ($P<0.001$). G, The association between LSD1 expression level and RFS in RCC patients ($P<0.001$). $^{*}P<0.05$.

Table 1 Association of LSD1 expression with clinicopathological characteristics in 358 ccRCC patients.

Characteristic		Patients		Tumoral LSD1 expression		*P* value
		n	%	Low	High	
All patients		358	100	134	224	
Gender						0.618[a]
	Male	254	70.9	93	161	
	Female	104	29.1	41	63	
Age (years)						0.107[a]
	≤55	178	49.7	74	104	
	>55	180	50.3	60	120	
TNM stage						0.006[a,*]
	I+II	341	95.3	133	208	
	III+IV	17	4.7	1	16	
pT stage						0.017[a,*]
	T1+T2	344	96.1	133	211	
	T3+T4	14	3.9	1	13	
pN stage						0.030[b,*]
	N0	349	97.5	134	215	
	N1	9	2.5	0	9	
pM stage						0.088[b]
	M0	352	98.3	134	218	
	M1	6	1.7	0	6	
Fuhrman grade						0.266[a]
	I+II	297	83	115	182	
	III+IV	61	17	19	42	
Tumor size (cm)						0.107[a]
	≤4	186	52	77	109	
	>5	172	48	57	115	

[a]Chi-square test.
[b]Fisher's exact test.
[*]$P<0.05$ indicates a significant association among the variables.

clinicopathological characteristics are summerized in Table 1. It was found that a total of 224 (62.6%) cases had high LSD1 staining scores (Supporting Information Table S2) for LSD1 expression, which was mainly present in the nuclei. Of note, LSD1 expression was statistically associated with tumor stage ($P=0.017$), lymph node metastasis ($P=0.030$), and also TNM stage ($P=0.006$) in these ccRCC patients. Representative images of LSD1 staining associated with individual TNM stages are shown in Fig. 1E. Prognostic analysis indicated that patients with high LSD1 expression exhibited shorter overall survival (OS) and recurrence-free survival (RFS) (Fig. 1F and G). Univariate and multivariate analysis also demonstrated that LSD1 expression was an independent prognostic factor of OS and RFS among ccRCC patients (Tables 2 and 3). These results show that LSD1 is associated with poor prognosis in ccRCC patients.

3.2. *Inhibition of LSD1 suppresses cell proliferation in RCC cell lines*

The results above suggest the potential role of LSD1 in the progression of ccRCC. We next wanted to address whether LSD1 intervention might result in therapeutic benefits. To this end, we examined the LSD1 protein level across a small panel of ccRCC cell lines composed of 786-O, ACHN, CAKI-1, A498 and 769-P cell lines. HK2 cells were used as a normal control. Immunoblotting showed that 786-O and CAKI-1 cell lines expressed the highest level of LSD1 among all the tested lines (Fig. 2A), and were used for further studies. We then knocked down LSD1 expression in 786-O and CAKI-1 using two independent siRNAs. Consistent with previous findings, downregulation of LSD1 induced the specific accumulation of H3K4me2. H3K9me2 and the total histone H3 was examined in controls (Fig. 2B). Importantly, dowregulation of LSD1 significantly decreased the growth of 786-O and CAKI-1 cell lines (Fig. 2C), suggesting that LSD1 is required for the proliferation of ccRCC cell lines.

To determine whether LSD1 downregulation caused cell growth retardation resulting from impaired demethylase activity, we treated 786-O and CAKI-1 cells with three reported LSD1 enzymatic inhibitiors, including SP2509, ORY1001 and tranylcypromine[28,29]; the anticancer effects in 786-O and CAKI-1 cell lines are shown in Supporting Information Fig. S2. SP2509, with the most potent impact on cell proliferation, was selected for the following studies. Cells were treated with various concentrations of SP2509 and methylation levels of H3K4 and H3K9 were examined. SP2509 treatment evidently increased H3K4me2 levels in both cell lines, without signicant change in H3K9me2 level (Fig. 2D). In line with the siRNA depletion experiment, SP2509 treatment significantly inhibited cellular proliferation in a time- and dose-dependent manner (Fig. 2E). We also did several tests of SP2509 inhibition on A498 and ACHN, which have low LSD1 expression. The result showed that the cell lines with higher LSD1 have a better inhibition rate of SP2509 (Supporting Information Fig. S3). Similar results were obtained using the colony formation assays, which showed that 50 nmol/L SP2509 treatment was sufficient to suppress colony formation in both 786-O and CAKI-1 cells (Fig. 2F).

The suppressed cell growth caused by anticancer drugs often results from the arrested cancer cycle progression or the promoted cell apoptosis. We examined the impact of LSD1 inhibition on cell cycle progression. The results demonstrated that LSD1 silencing significantly incresed the ratio of G1-phase cells, which implied an effect of G1/S arrest (Fig. 2G and H). The same result was obtained using SP2509 treatment (Fig. 2I and J). Meanwhile, the occurrence of apoptosis upon both siRNA silencing and SP2509 inhibition was not evident (Supporting Information Figs. S4 and S5). These results suggest that cell growth inhibition resulted from delayed cell cycle progression. As cell cycle-related cell growth delay is known to be reversible, we carried out a drug-withdraw experiment by disposition with SP2509 in 786-O and CAKI-1 cells for 24 h, and replacing with the culture medium afterwards. Cell numbers were counted every 24 h and the results indicated that initially arrested ccRCC cells were liberated to continue proliferation after treatment withdrawal, and achieved the similar cell counts when reaching 96 h post treatment (Fig. 2K and L). In summary, the above results suggest that the impairment of LSD1 enzymatic activity inhibits the growth of ccRCC cells *via* arresting the cells at G1/S phase.

3.3. *LSD1 inhibition results in G1/S phase arrest via the upregulation of P21 signal pathway*

To further identify the molecular signaling pathways that might be involved in mediating the impact of LSD1 on cell cycle regulation, we performed RNA-seq analysis of 786-O cells after 24-h treatment with SP2509. The heat-map showed the overall change of the expression pattern, with upregulated genes indicated in red and downregulated

Table 2 Summary of univariate and multivariate Cox regression analysis of OS duration in all ccRCCs.

Variable		Univariate analysis			Multivariate analysis		
		HR	(95% CI)	P^*	HR	(95% CI)	P^*
LSD1 in cancer tissues							
	Low	1			1		
	High	4.684	2.445–8.973	< 0.001*	3.571	1.846–6.908	< 0.001*
Gender							
	Male	1					
	Female	0.814	0.482–1. 375	0.442			
Age (years)							
	≤ 55	1			1		
	>55	1.736	1.081–2.788	0.022*	1.265	0.766–2.090	0.358
TNM stage							
	I+II	1			1		
	III+IV	10.185	5.637–18.403	< 0.001*	0.537	0.101–2.866	0.467
pT stage							
	T1+T2	1			1		
	T3+T4	12.239	6.503–23.033	< 0.001*	4.884	1.040–22.937	0.044*
pN stage							
	N0	1			1		
	N1	18.838	8.851–40.093	< 0.001*	5.569	1.786–17.360	0.003*
pM stage							
	M0	1			1		
	M1	11.539	4.953–26.879	< 0.001*	1.614	0.508–5.124	0.417
Fuhrman grade							
	I+II	1			1		
	III+IV	3.243	2.013–5.225	< 0.001*	2.470	1.496–4.079	< 0.001*
Tumor size (cm)							
	≤ 4	1			1		
	>4	5.573	3.058–10.157	< 0.001*	3.641	1.944–6.819	< 0.001*

HR, hazard ratio; 95% CI, 95% confidence interval.
*$P < 0.05$ indicates a significant association among the variables.

genes in green (Fig. 3A). The cufflinks software program was applied to identify genes with significant alteration of expression ($P < 0.05$) in response to SP2509 treatment (Fig. 3B). Further Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed that most of the altered genes were enriched in the pathways promoting cancer malignacy and indicated the potential role of LSD1 in promoting RCC progression. Several differentially expressed images of the GO and KEGG analysis results are also supplied in Supporting Information Fig. S6.

Among the affected pathways, we noticed that the P53 pathway was influenced by SP2509 treatment (Fig. 3C). As the P53 pathway is known to respond to DNA damage or other insults in keeping a balance of cell cycling and cell death[30] and regulates the G1/S transition, consistent with the cellular phenotype upon LSD1 inhibition observed above, we chose to focus on this pathway. We examined the expression of P53 and P21, two key components in this pathway after SP2509 treatment or siRNA transfection. It was revealed that inhibition of LSD1 was able to elevate P21 protein expression, while the P53 level was barely affected (Fig. 3D and E). We also examined the mRNA level of *P21* following LSD1 knockdown in both 786-O and CAKI-1 cells and found that the *P21* mRNA level was significantly increased compared to negative controls (Fig. 3F). These results suggest that P21 was transcriptionally upregulated upon LSD1 inhibition.

As P53, the direct upstream effector of P21 was not affected, we speculated that LSD1 might possibly modulate the H3K4me2 status of the *CDKN1A* gene. To test this possibility, we performed a ChIP-qPCR assay using anti-H3K4me2 antibody and primers specifically targeting the *P21* gene promoter (Supporting Information Fig. S7). The results showed the enrichment of H3K4me2 modification on the promoter of the *CDKN1A* gene, which encodes the P21 protein, upon *LSD1* knockdown in both 786-O and CAKI-1 cell lines. Consistent with this, the immunoprecipitate with anti-LSD1 antibody showed a decrease of LSD1 binding to the *CDKN1A* promoter following *LSD1* knockdown (Fig. 3G). These results suggest that LSD1 inhibition increased the H3K4 methylation on the *CDKN1A* promoter which in turn led to the transcriptional activation of *P21* in ccRCC cells. What's more, P21 expression in clinical samples between the subgroups of LSD1 with high/low expression is shown in Supporting Information Fig. S8. Expression of P21 was relatively low in RCC samples, and samples with higher LSD1 expression always have lower P21 expression.

It is generally acknowledged that P21 binds to and inhibits the activity of cyclin CDK4/6 complexes and functions as a checkh-point regulator. We examined *CDK4* and *CDK6* mRNA levels and detected an apparent decrease following *LSD1* silencing (Fig. 3H and I). The protein levels of CDK4 and CDK6 were decreased consistently following SP2509 or LSD1 siRNA treatment (Fig. 3J and K). Taking together, our findings indicate that LSD1 inhibition

Table 3 Summary of univariate and multivariate Cox regression analysis of RFS duration in all ccRCCs.

Variable		Univariate analysis			Multivariate analysis		
		HR	(95% CI)	P^*	HR	(95% CI)	P^*
LSD1 in cancer tissues							
	Low	1			1		
	High	3.430	1.881–6.252	P < 0.001*	2.939	1.596–5.411	0.001*
Gender							
	Male	1					
	Female	0.888	0.531–1. 483	0.649			
Age (years)							
	≤ 55	1					
	>55	1.426	0.898–2.263	0.132			
TNM stage							
	I+II	1			1		
	III+IV	7.710	4.135–14.379	< 0.001*	0.536	0.096–3.008	0.479
pT stage							
	T1+T2	1			1		
	T3+T4	8.564	4.369–16.789	< 0.001*	3.953	0.817–19.120	0.087
pN stage							
	N0	1			1		
	N1	14.814	6.675–32.879	< 0.001*	5.348	1.591–17.980	0.007*
pM stage							
	M0	1			1		
	M1	8.113	2.952–22.291	< 0.001*	1.171	0.340–4.033	0.802
Fuhrman grade							
	I+II	1			1		
	III+IV	3.079	1.900–4.987	< 0.001*	2.253	1.368–3.711	0.001*
Tumor size (cm)							
	≤ 4	1			1		
	>4	6.618	3.565–12.286	< 0.001*	4.978	2.630–9.423	< 0.001*

HR, hazard ratio; 95% CI, 95% confidence interval.
*$P < 0.05$ indicates a significant association among the variables.

results in G1/S phase arrest *via* the upregulation of P21 signal pathway.

3.4. *SP2509 inhibits ccRCC xenograft tumor growth in vivo*

Based on the above findings, we were encouraged to evaluate the therapeutic potential of the LSD1 selective inhibitor SP2509 in animal models. We evaluated the *in vivo* anti-tumor activity of SP2509 in female athymic mice. Following the subcutaneous engraftment of 786-O and CAKI-1 cells in the flank region, mice were treated with SP2509 in a dose of 15 mg/kg for 4 weeks and tumor volumes were successively monitored and measured twice a week. The results showed that the average tumor volume in SP2509-treated group was significantly smaller when compared with the control group ($P<0.05$ in 786-O mice, $P<0.01$ in CAKI-1 mice) (Fig. 4A and B). However, the average body weight between the two groups was comparable, which indicated that SP2509 exerted its anti-tumor effects without severe toxicity (Fig. 4C). When euthanized, xenograft tumors were harvested and weighed, and the average tumor weight was clearly lower in the drug-treated groups ($P<0.01$ both in 786-O and CAKI-1 mice, Fig. 4D). We also evaluated LSD1 and P21 expression level in the SP2509-treated group and control group by IHC and verified that SP2509 treatment was associated with obviously increased P21 expression (Fig. 4E). Therefore, our data strongly suggest that high LSD1 inhibition would probably enhance RCC tumor growth *in vivo* and could be reversed by LSD1 inhibition, which is consistent with our previous *in vitro* findings. Taken together, we provide a schematic diagram to help illustrate the molecular mechanisms in which LSD1 participates in P21 signal modulation, how it regulates RCC cell proliferation and ultimately serves as a novel potential therapeutic target (Fig. 4F).

4. Discussion

Despite the intensive research in mechanisms associated with RCC tumorigenesis and progression, the treatment options for patients with advanced RCC remain limited. Recent studies have shown that LSD1, the first identified histone lysine-specific demethylase, plays an important role in various human malignancies, suggesting that inhibition of LSD1 may be an attractive strategy for cancer treatment. However, to our knowledge, few studies have explored either the regulatory mechanism of LSD1 or the therapeutic potential of targeting LSD1 in kidney cancer. Data-mining across the GENT database indicated higher-than-normal expression of LSD1 in kidney cancer, which was validated at both the mRNA

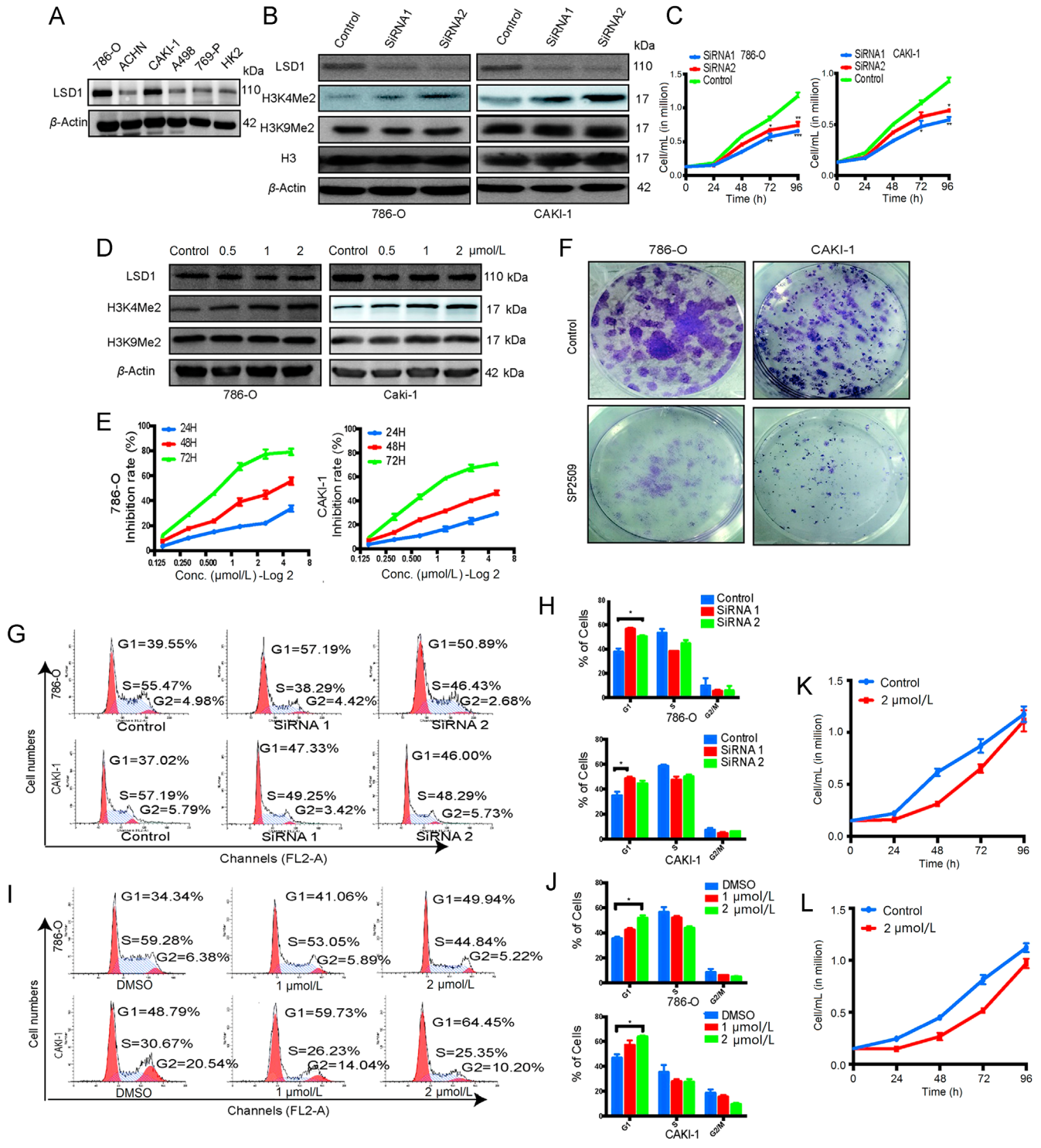

Figure 2 Inhibition of LSD1 suppressed the proliferation in RCC cell lines. A, Expression of LSD1 in RCC cell lines analyzed by Western blot, with *β*-actin used as a loading control. 786-O and CAKI-1 cells show high LSD1 expression among RCC cell lines. B, Western blot analysis shows increased expression of H3K4me2 when LSD1 expression was knocked down, while H3k9me2 and H3 show no significant change. C, A cell counter was used to evaluate the knock-down of LSD1 on the proliferation of 786-O and CAKI-1 at indicated time points, results are expressed as mean ± SD. D, 786-O and CAKI-1 cells were treated with the indicated concentration of SP2509 for 24 h, and Western blotting was used to determined the downstream methylation target. E, SP2509 induced cell death in 786-O and CAKI-1 in a dose- and time-dependent manner. F, colony formation of 786-O and CAKI-1 after 50 nmol/L SP2509 treatment compared with negative control. G, knock-down of LSD1 significantly induced cell cycle G1/S arrest in 786-O and CAKI-1 cells. H, summarized data regarding the cell numbers at each cell cycle phase after LSD1 knock-down. I, SP2509 significantly induced cell cycle G1/S arrest in 786-O and CAKI-1 cells. J, summarized data regarding the cell numbers of each cell cycle phase after drug treatment. K–L, withdrawal experiments from SP2509 after a 24 h treatment of 786-O and CAKI-1 cells with over time. Error bars are ± SD of three independent experiments $^{*}P<0.05$, $^{**}P<0.01$.

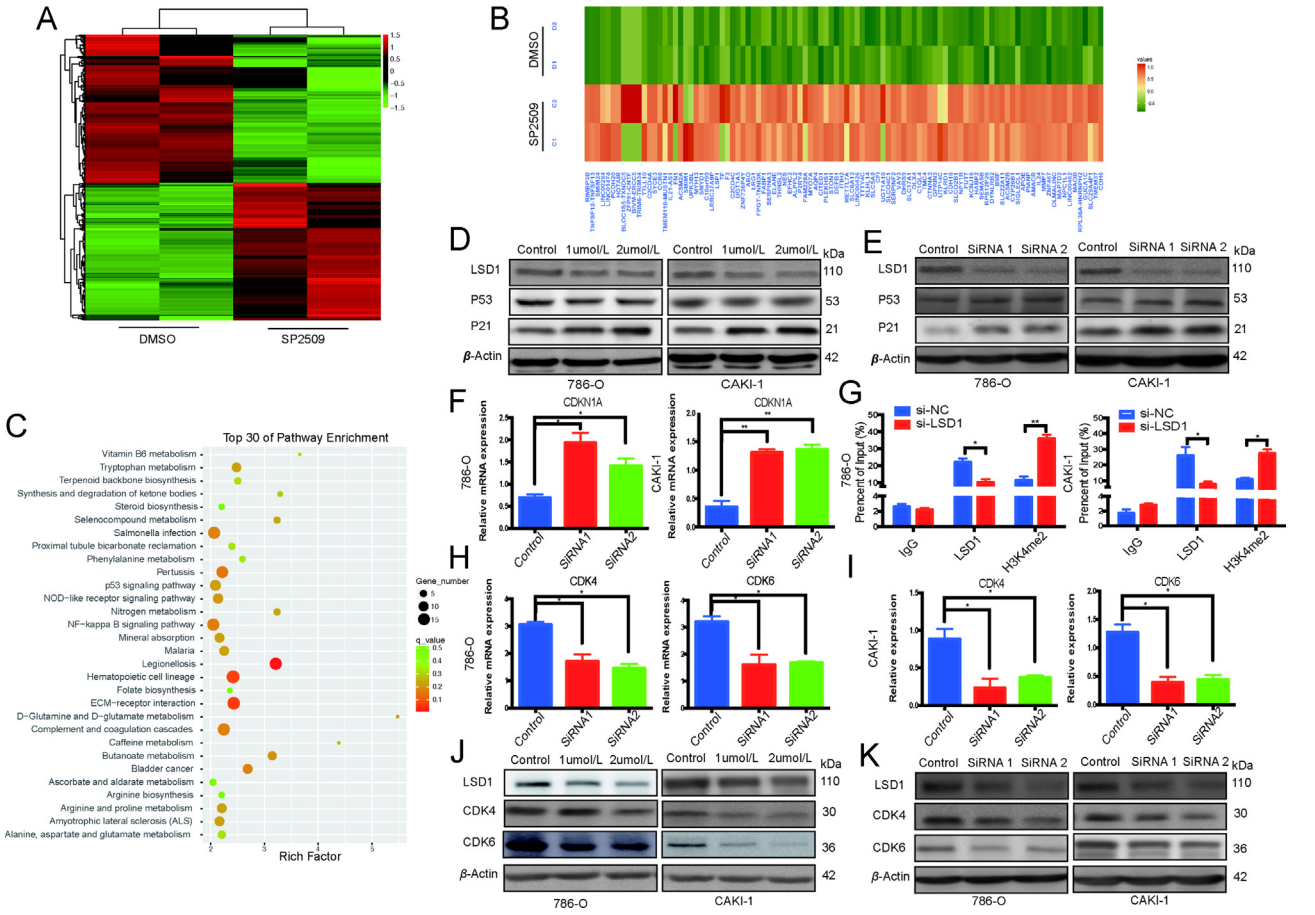

Figure 3 Inhibition of LSD1 suppressed cell proliferation by inducing cell cycle G1–S arrest by upregulating the P21 signaling pathway in RCC cells. A, heat map of total differential gene expression in 786-O cells treated with DMSO control and 5 μm SP2509 after 24 h. B, genes were selected by using the Cufflinks software program with $P<0.05$, and upregulated genes are in red. C, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed to explore the top 30 pathways enrichment. D. The antibodies against P53, P21 and *β*-actin were used to determine the effect of SP2509 on the activities of cell cycle signaling. E, antibodies against P53, P21 and *β*-actin were used to determine the knock-down effect of LSD1 on cell cycle signaling. F, expression of *P21* was determined by qRT-PCR after si-LSD1 was transfected into 786-O and CAKI-1 cells. G, ChIP analysis was performed on the *P21* promoter regions using anti-H3K4me2 and anti-LSD1. Enrichment was calculated relative to the input controls. H, expression of CDK4 and CDK6 was determined by qRT-PCR after si-LSD1 was transfected into 786-O cells. I, expression of CDK4 and CDK6 was determined by qRT-PCR after si-LSD1 was transfected into CAKI-1 cells. J, antibodies for CDK4, CDK6 and *β*-actin were used to determine the effect of SP2509 on the activities of P21 downstream pathway components. K, antibodies to CDK4, CDK6 and *β*-actin were used to determine the effect of LSD1 silencing on the activities of P21 downstream pathway components. The data are presented as mean ± SD of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$.

and protein levels with fresh samples from our institute. Notably, TMA screening, which contained 358 ccRCC cases, demonstrated that overexpression of LSD1 was associated with shorter OS and RFS, suggesting that LDS1 expression might serve as an independent prognostic factor in ccRCC patients.

It has been proven that LSD1 contributes to the formation of transcription repression complexes such as CoREST and NuRD[31], in which LSD1 may inhibit the targeted gene transcription *via* demethylation of H3K4me or H3K4me2. A series of *in vitro* and *in vivo* experiments were performed in this study to explore the role of LSD1 in ccRCC. It was found that inhibition of LSD1, either by siRNA silencing or SP2509 blockade significantly suppressed cell proliferation, mediated by G1/S cell cycle arrest, along with the accumulation of H3K4me2. These results are consistent with a previous report in non-small cell lung carcinomas, demonstrating that downregulation of LSD1 suppressed cell proliferation and migration capacity[32]. In our mouse model study, SP2509 treatment induced a significant decrease of tumor volume and tumor weight without obvious toxicity. Both the *in vitro* and *in vivo* results support the notion that LSD1 is an important regulator in RCC proliferation.

It has been well known that the G1–S transition in mammalian cells is controlled by cyclin/cyclin-dependent kinases (CDKs) and associated cyclin-dependent kinase inhibitors (CKIs). Furthermore, dysregulation of CKIs is a common feature in the course of tumor development across different cancer types[33]. P21 (also known as CIP1, WAF1 and cyclin-dependent kinase inhibitor 1 A), encoded by the *CDKN1A* gene located on chromosome 6p21.2, is a well-known tumor suppressor that regulates cell proliferation[34], and represents one of the most important targets in the P53 signaling pathway, as it inhibits downstream CDK4/6 kinases activity[35]. Other reports have reviewed the modulating role of P21 in the cell cycle and mitosis and discovered that P21 was downregulated across various types of cancers[36,37]. Our study showed that LSD1 knockdown or LSD1 inhibition induced the accumulation of P21 protein as well as G1/S cell cycle arrest in ccRCC cell lines. ChIP assays using the anti-LSD1

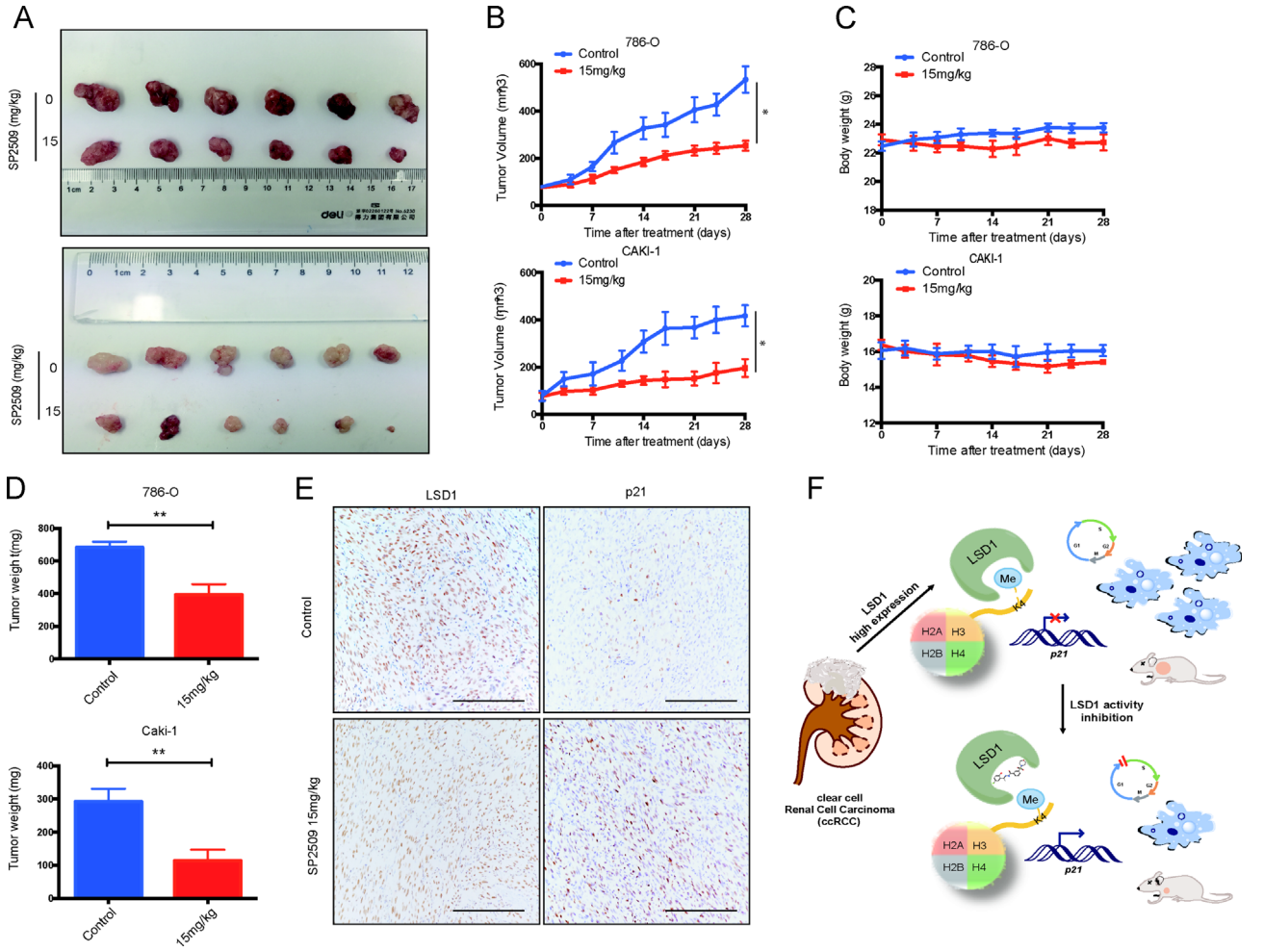

Figure 4 Treatment of RCC cells with SP2509 inhibited tumor cell growth *in vivo*. A, xenograft tumor model of RCC cell lines with (15 mg/kg) or without SP2509, representative photographs 28 days after injection (Upper, 786-O group and lower, CAKI-1 group). B, tumor volume measurement at the indicated time points. C, body weight of the mice at the indicated points. D, bar graph of the tumor weight comparing the control group with SP2509 treatment. E, IHC of tissue harvested from the mouse tumor model stained for LSD1 and P21, bar: 200 μm. F, schematic diagram showing that LSD1 mediates ccRCC cell proliferation. (I) LSD1 expression in ccRCC samples is high and it suppresses H3K4 methylation, inducing ccRCC cell proliferation through P21 signaling dysfunction. (II) LSD1 inhibition restores H3K4 methylation, inducing G1/S cell cycle arrest by increasing the level of check-point regulator P21. $^{*}P<0.05$, $^{**}P<0.01$.

and anti-H3K4me2 antibodies suggested that LSD1 regulates *CDKN1A* gene expression *via* modulating the demethylation of the K4 amino acid. Of interest, we also observed that CDK4/6 was significantly suppressed by LSD1 inhibition. However, we still have not found the detailed mechanism by which CDK4/6 proteins downregulate after LSD1 inhibition, which appears to be independent of P21 upregulation. These findings imply that LSD1 coordinates P21 signaling *via* multiple mechanisms.

Several studies have recently shown that LSD1 participates in chromatin remodeling *via* modifying the regulation of histone methylation. Liu et al.[38] reported that LincRNAFEZF1-AS1 repressed P21 expression in the manner of LSD1-mediated H3K4me2 demethylation and promoted gastric cancer progression. LSD1 overexpression has been discovered to be involved in many processes of malignancies, such as proliferation, invasion and cell cycle acceleration[39,40]. According to the GO classification in our study, we showed that the LSD1 inhibitor mainly decreased the cellular process and reduced antioxidant activity. Several pathways that involve energy metabolism in ccRCC were shown to be affected by the downregulation of LSD1 as well. These findings suggest that LSD1 may also have a valuable role in energy metabolism, and further work is needed to explore the potential mechanism of LSD1 in metabolic modulation.

In conclusion, our study provides the first evidence that LSD1 is overexpressed in ccRCC tissues, which is also significantly associated with poor clinical outcomes. Furthermore, inhibition of LSD1 expression or activity induces G1/S arrest and inhibition of cell proliferation *in vitro*, possibly *via* modulating the P21 signaling pathway. *In vivo* experiments further verify that antagonist targeting of LSD1 inhibits growth of engrafted renal tumors, suggesting that LSD1 may be a novel molecular target for new drug development for advanced ccRCC. Further research is still needed to explore in detail the mechanism by which demethylation and the P21 pathway participate in ccRCC development.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.apsb.2018.10.006.

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