SPOP promotes tumor progression via activation of β-catenin/TCF4 complex in clear cell renal cell carcinoma

WENCAI ZHAO[1*], JIANCHENG ZHOU[1*], ZHUO DENG[2], YANG GAO[1] and YONGYI CHENG[1]

Departments of [1]Urology and [2]Gynecology, Shaanxi Provincal People's Hospital, Xi'an, Shaanxi 710068, P.R. China

Received May 2, 2016; Accepted June 22, 2016

DOI: 10.3892/ijo.2016.3609

Abstract. Renal cell carcinoma (RCC) is the most common type of kidney cancer, about one third of the cases are diagnosed at advanced stages with metastases and effective treatments for metastatic RCC are lacking. The molecular events supporting RCC progression remain poorly understood. SPOP, an E3 ubiquitin ligase component, was recently showed to sufficiently promote RCC tumorigenesis, however, other potential functions of SPOP in RCC have not been studied. In the present investigation, by assessing the immunohistochemical staining of SPOP in urological tumors, we found the protein was highly expressed in RCC, in particular, it was specifically expressed in clear cell RCC. cDNA microarray data showed that SPOP mRNA level was significantly increased in clear cell RCC compared to normal kidney tissues, which might be the result of the abnormal DNA copy number of this gene. More interestingly, SPOP was positive in tumors with local invasion or metastasis, and it was associated with tumor recurrence-free survival of clear cell RCC patients. Further *in vitro* assays demonstrated that SPOP drove RCC epithelial-mesenchymal transition (EMT) and promoted cell invasion. Mechanistically, SPOP enhanced β-catenin protein expression as well as its nuclear translocation, and elevated TCF4 expression. Both β-catenin and TCF4 upregulated the critical EMT-inducing transcription factor ZEB1, which functioned as an effector of β-catenin/TCF4 signaling in RCC invasion. These data identified SPOP as a new marker and prognostic factor for clear cell RCC, and its functions provide new insight into the molecular mechanisms of RCC progression, in which SPOP appears to be an EMT activator.

Correspondence to: Dr Jiancheng Zhou, Department of Urology, Shaanxi Provincal People's Hospital, 256 West Youyi Road, Xi'an, Shaanxi 710068, P.R. China
E-mail: 26675085@163.com

*Contributed equally

Key words: SPOP, renal cell carcinoma, epithelial-mesenchymal transition, β-catenin, TCF4

Introduction

Renal cell carcinoma (RCC) is the most common type of kidney cancer, >200,000 new cases and 100,000 deaths are estimated to occur worldwide each year (1). Up to 30% of RCC patients present with metastases at the time of diagnosis and nearly half of the rest will subsequently develop metastases in their course. When metastasis occurs, it is largely incurable, with a very poor 5-year survival rate (2). RCC is highly resistant to chemotherapy and radiotherapy, immunotherapies such as interleukin-2 and interferon α are once used as first-line treatments for metastatic RCC (mRCC), however, the response rates are extremely low (3). Newly developed targeted-therapies based on the understanding of molecular mechanisms of RCC progression make significant improvements over immunotherapies for mRCC. Unfortunately, <40% of patients have response to targeted-therapies and nearly all patients will eventually develop resistance (4,5). It appears that a therapeutic ceiling has been reached for mRCC, thus, it is important to comprehensively study the mechanisms of how RCC develops metastasis, and explore promising therapeutic approaches for this disease.

SPOP, a BTB/POZ domain containing speckle-type POZ protein, was first identified as a component for the E3 ubiquitin ligase (6). In *Drosophila*, D-SPOP (ortholog of human SPOP) has been shown to promote the ubiquitination and degradation of Cubitus interruptus (Ci) in the Hedgehog pathway, and JNK phosphatase puckered (Puc) in the tumor necrosis factor (TNF) pathway, respectively (7-9). In human, SPOP has been recently shown to mediate ubiquitination of the death domain-associated protein (Daxx) (10), the polycomb group protein BMI-1, the histone variant MacroH2A (11), and the transcription factor Gli (9).

In RCC, hypoxia-inducible factor (HIF) and mammalian target of rapamycin (mTOR) pathways are considered as the most predominant pathways controlling RCC development and progression (12,13), and therapies targeting these two pathways have brought clinical benefits to mRCC (14). A more recent study shows that SPOP is a direct target of HIF, and cytoplasmic SPOP promotes RCC tumorigenesis through the ubiquitination and degradation of multiple regulators of cellular proliferation and apoptosis, including the tumor suppressor PTEN, ERK phosphatases DUSP7, the proapoptotic molecule Daxx, and the Hedgehog pathway transcription

factor Gli2 (15). However, other potential functions of SPOP in RCC have not been studied. In this study, we aim to determine whether SPOP promotes invasion and metastasis in RCC.

Materials and methods

Human RCC specimens and immunohistochemistry staining. Forty-seven human RCC and 11 matched normal kidney specimens were obtained from patients who underwent surgical resection with the approval of the Institutional Review Board (IRB). Immunohistochemistry was performed as described previously (16). Briefly, paraffin-embedded sections were subjected to deparaffinization, rehydration, and heat-induced antigen retrieval. After blocking of endogenous peroxidase with 3% hydrogen peroxide, sections were subsequently incubated with primary SPOP antibody (Santa Cruz Biotechnology), horseradish-peroxidase-labeled dextran polymer (Dako EnVision™) and developed with 3,3'-diaminobenzidine chromogen followed by counter staining with hematoxylin. All stains were assessed by an independent pathologist according to the histologic scoring system (H-score) based on the product of staining intensity (0, no staining; 1, weak; 2, moderate; and 3, strong) and percentage of stained cells (0, 0%; 1, 1-30%; 2, 31+70%; and 3, 71-100%). The expression of SPOP in each tissue was considered either negative (H-score, <2) or positive (H-score, >2).

Cell culture. Human normal kidney cell HK-2, RCC cells 786-0, A498, RCC4 and 769-P were maintained in RPMI-1640 medium (Gibco) supplied with 10% fetal bovine serum (FBS), RCC cells ACHN, CAKI-1, CAKI2 and A498 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplied with 10% FBS. All the cells were cultured in a humidified incubator containing 5% CO_2 at 37˚C.

cDNA constructs, siRNA and transfection. Human SPOP cDNA cloned into pDONR221 vector was obtained from the DNASU Plasmid Repository. Control siRNA (si-NC) and siRNA specifically targeting SPOP (si-SPOP) were from Guangzhou RiboBio Co., Ltd. For transfections, $2x10^5$ cells were seeded in 6-well plate and cultured overnight, SPOP plasmids or siRNAs were transfected into cells by Xfect™ transfection reagent (Clontech) according to the manufacturer's instructions. Twenty-four hours (h) after the transfection, cell protein or RNA was collected for further assays.

Transwell invasion assay. Matrigel-coated Transwell chambers were applied to examine RCC cell *in vitro* invasive ability. RCC cells were pre-transfected with the indicated plasmids or siRNAs, 100 μl 0.5% FBS medium of $3x10^4$ cell suspension was then planted into the upper chamber, and 600 μl of 10% FBS medium was supplied to the lower chamber. Cells were cultured at 37˚C in 5% CO_2 for 24 h. Invaded cells onto the lower surface of the upper chambers were stained with 0.5% crystal violent (Sigma) and photographed and counted.

Immunofluorescence. Microslide cultured cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 and blocked with 5% bovine serum albumin. Cells were incubated with β-catenin primary antibody (Cell Signaling Technology) overnight at 4˚C and subsequently incubated with AlexaFluor 488-conjugated secondary antibody (Sigma) for 1 h at room temperature, followed by nuclear staining with 4,6-diamidino-2-phenylindole and fluorescence was visualized by fluorescence microscopy (Olympus Optical Co.)

Reverse transcriptional (RT) real-time PCR. Cell total RNA was extracted with RNeasy mini kit (Qiagen) and reverse transcribed with cDNA synthesis kit (Invitrogen). Real-time PCR analysis was set up with SYBR Green qPCR Supermix kit (Invitrogen) supplied with commercial primers specific for the indicated genes, and carried out in the iCycler thermal cycler (Bio-Rad). The relative level of mRNA expression of each gene was determined by normalizing with an internal control gene GAPDH.

Western blotting. Western blotting was performed as previously described (17). Cells were first lysed and total proteins were collected, equivalent amounts of protein were separated on 10% NuPAGE Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes. Membranes were blocked with 3% skim-milk (w/v), and incubated with primary antibodies overnight at 4˚C. After washing, membranes were incubated with appropriate secondary antibodies conjugated with horseradish peroxidase and signals were then detected by chemiluminescence (Pierce). Primary SPOP, vimentin, pan-cytokeratin (Pan-CK), TCF4 and GAPDH antibodies were purchased from Santa Cruz Biotechnology; E-cadherin and α-SMA antibodies were from BD Biosciences, ZEB1 and MMP-2 antibodies were from Cell Signaling Technology.

Bioinformatic and statistical analyses. The RNA-sequencing-based mRNA expression data for SPOP, TCF4, ZEB1 genes and the reverse phase protein array-based protein expression data for β-catenin of human clear cell RCC samples were all retrieved from The Cancer Genome Atlas (TCGA) Data Portal (18). Gene microarray data for SPOP of human normal kidney and clear cell RCC tissues were retrieved from the GEO datasets (GSE14994, GSE781 and GSE15641). SPOP gene microarray data and DNA copy number data of multiple types of cancer cell lines were retrieved from the Cancer Cell Line Encytopedia (CCLE) datasets. The Kaplan-Meier analysis (long-rank test) was performed to analyze recurrence-free survival. Pearson's correlation coefficient was used to test the association between genes. Data from *in vitro* assay are presented as the mean ± SEM from three independent experiments, and the differences between two groups were compared by Student's two-tail t-test. All statistical analyses were performed by GraphPad Prism6 (GraphPad Software).

Results

SPOP is highly expressed in clear cell RCC. Previous studies suggest that overexpression of SPOP may lead to dysregulation of pathways involved in tumorigenesis (8,9). We assessed SPOP expression in urological tumors including prostate cancer, bladder cancer, RCC and normal kidney tissues by immunohistochemistry. Interestingly, we found SPOP was negative in prostate cancer, bladder cancer and normal kidney tissues, but it was highly expressed in RCC tissues (Fig. 1A).

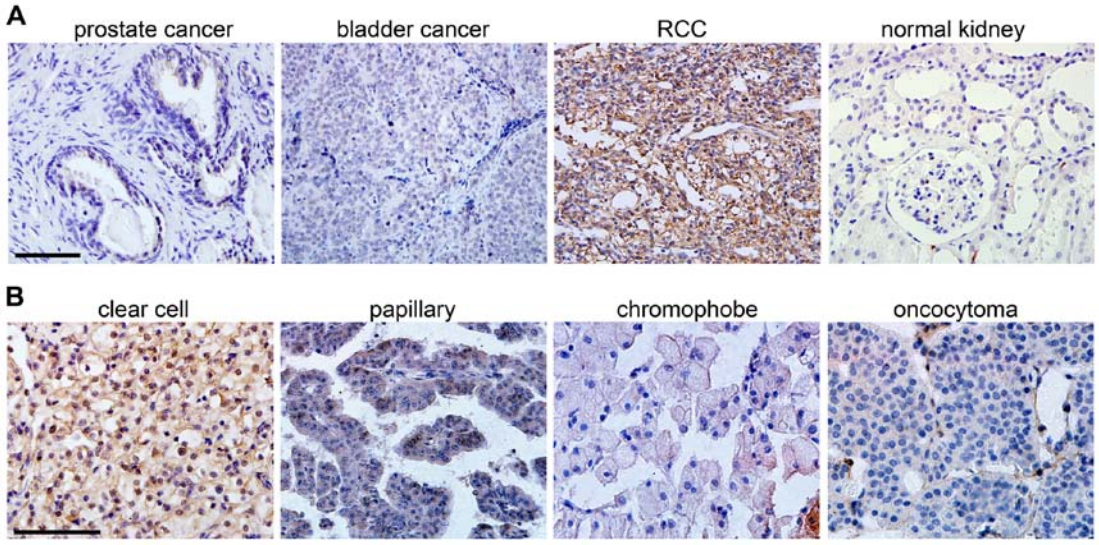

Figure 1. SPOP is specifically expressed in clear cell RCC. (A) Immunohistochemistry staining of SPOP in human prostate cancer, bladder cancer, RCC and normal kidney tissues. (B) Immunohistochemistry staining of SPOP in different subtypes of RCC tissue. All scale bars represent 100 μm.

Table I. SPOP IHC staining in normal and clear cell RCC tissues.

	SPOP		
	Positive (%)	Negative (%)	P-value
Normal tissues	2 (18)	9 (82)	<0.001
Clear cell RCC	39 (83)	8 (17)	

RCC is a heterogeneous group of tumors with distinct histological subtypes, including clear cell, papillary, chromophobe, and other rare subtypes in addition to oncocytoma (19). When RCC subtypes were stratified, we found that the papillary, chromphobe or oncocytoma RCC were weak or negative for SPOP, but clear cell RCC was positively stained with this protein (Fig. 1B). In total we analyzed 47 clear cell RCC and matched 11 normal kidney tissues, and the results showed that 83% clear cell RCC were positive for SPOP, while only 18% normal tissues were positive (Table I). This indicates that SPOP is highly expressed in clear cell RCC and may serve as a specific biomarker for this type of RCC.

In addition to the determination of SPOP protein status in clear cell RCC, we checked SPOP mRNA expression by analysis of gene microarray data of normal kidney and clear cell RCC from the GEO datasets. Results from three independent datasets consistently showed that SPOP mRNA was significantly upregulated in clear cell RCC compared to normal kidney (Fig. 2A). To further explore whether SPOP upregulation is due to genomic abnormality, we analyzed the association of SPOP mRNA level and its DNA copy number in multiple types of cancer cell lines including 1,014 samples from the CCLE datasets, and found there was a positive correlation between SPOP mRNA level and its DNA copy number (Fig. 2B), and in RCC cell lines, a similarly positive correlation was also found (Fig. 2C). These data suggest that the upregulation of SPOP in RCC may be due to the genomic variation.

Table II. SPOP IHC staining in clear cell RCC with local invasion/metastasis.

	SPOP	
	Positive (%)	Negative (%)
Tumor stage		
T1-2	17 (70)	7 (30)
T3-4	22 (96)	1 (4)
Lymph nodes		
N0	28 (80)	7 (20)
N1	11 (92)	1 (8)
Metastasis		
M0	31 (79)	8 (21)
M1	8 (100)	0 (0)

SPOP is associated with progressive clear cell RCC. Previous studies indicate that SPOP plays important roles during tumor cell apoptosis and proliferation (7,10,15), we further investigated the potential functions of SPOP in tumor progression. The expression of SPOP in human clear cell RCC with local invasion (tumor cell invaded into perirenal fat, renal capsule or regional lymph node) was detected by immunohistochemistry, notably, the results showed that almost all the RCC with local invasion were SPOP-positive (Fig. 3A). We compared SPOP expression in RCC with different pathological stages according to the 2010 AJCC TNM classfication (20), and found RCC in T3-4 stages (primary tumors with local invasion) showed much high frequency of SPOP positive staining compared to RCC in T1-2 stages (without local invasion). Similarly, RCC with lymph node invasion (N1) or distant metastasis (M1) showed very high frequency of SPOP positive staining compared to RCC in N0 (without lymph node invasion) or M0 (without distant metastasis) (Table II). These date suggest SPOP is associated with clear cell RCC invasion

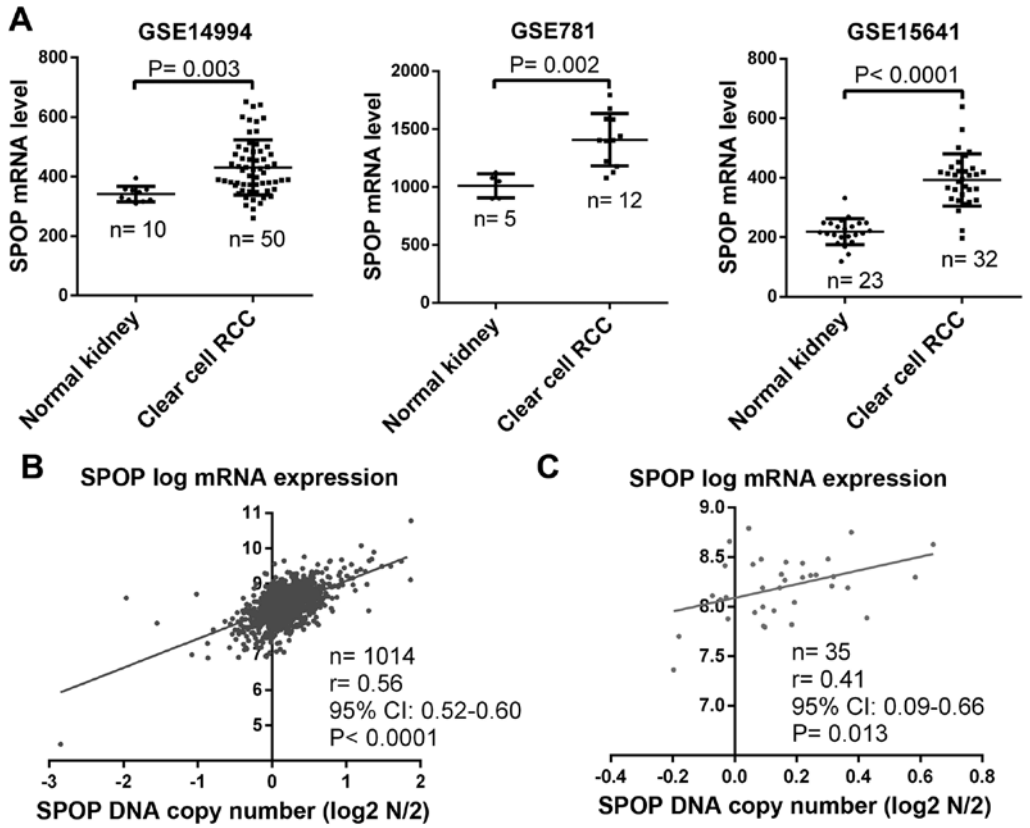

Figure 2. SPOP is significantly upregulated in clear cell RCC. (A) SPOP mRNA expression (cDNA microarray) in human normal kidney and clear cell RCC tissues from the GEO datasets. The serial number of each dataset, and the number of sample included for study in each dataset is described. Student's t-test was used to analyze the difference in SPOP levels between normal and RCC tissues. (B) Pearson correlation analysis of SPOP DNA copy number and mRNA level in multiple types of cancer cell lines from the CCLE datasets. (C) Pearson correlation analysis of SPOP DNA copy number and mRNA level in clear cell RCC cell lines from the CCLE datasets.

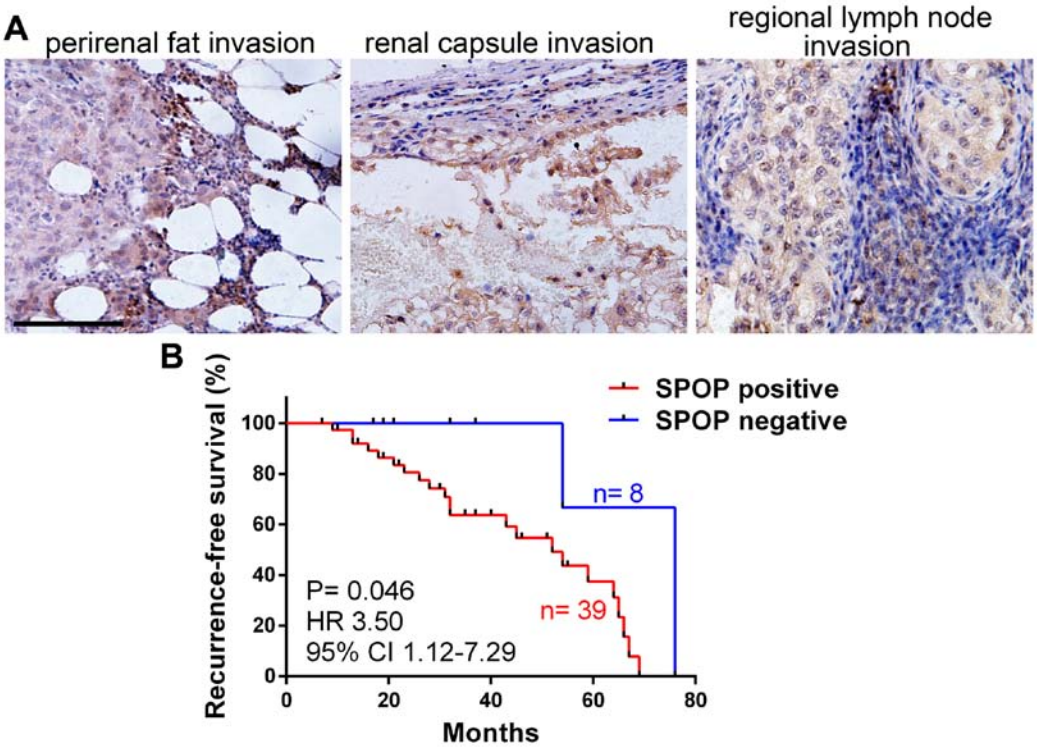

Figure 3. SPOP is associated with clear cell RCC invasion and poor survival. (A) Immunohistochemistry staining showing SPOP is positively expressed in human RCC with local invasion. (B) Kaplan-Meier analysis (log-rank test) showing the association between SPOP expression and recurrence-free survival of patients with clear cell RCC.

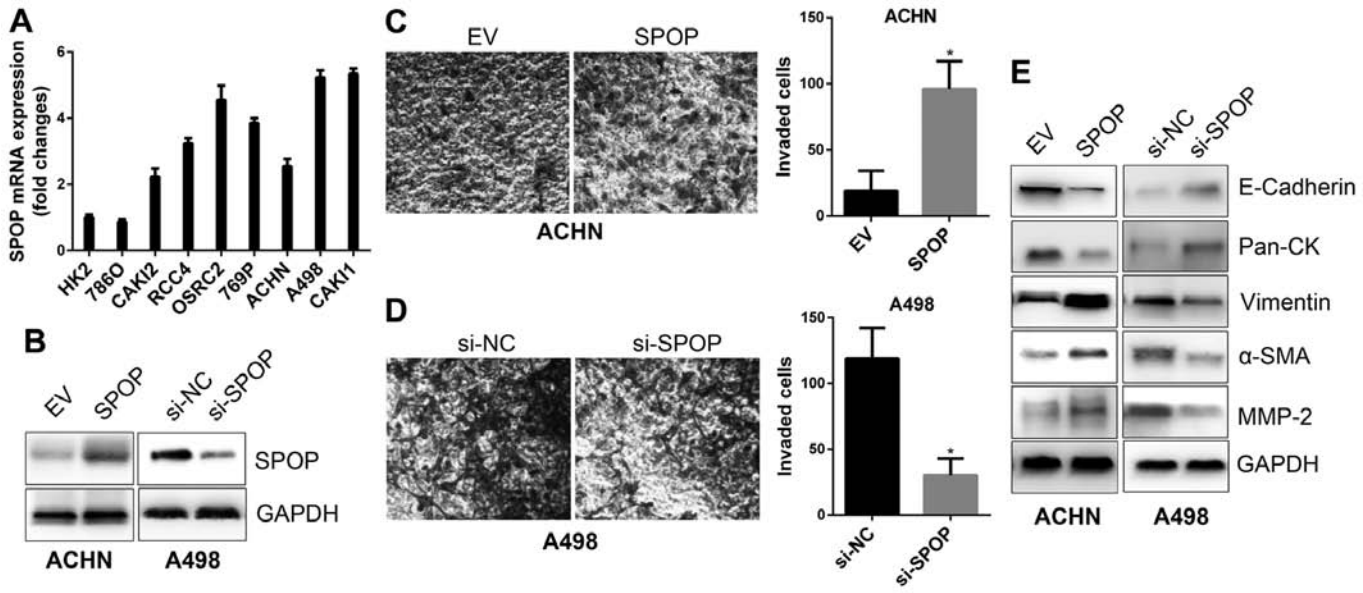

Figure 4. SPOP promotes RCC cell invasion and epithelial-mesenchymal transition (EMT). (A) Profiles of SPOP mRNA in different RCC cells by RT-PCR. (B) ACHN cells were transfected with empty vector (EV) or SPOP overexpression plasmid (SPOP), A498 cells were transfected with negative control siRNA (si-NC) or SPOP siRNA (si-SPOP). SPOP expression in cells were determined by western blotting, and GAPDH was used as loading control. (C and D) Transwell assays showing cell invasion of ACHN transfected with EV or SPOP plasmids, and A498 transfected with si-NC or si-SPOP. *P<0.05 compared to control. (E) Western blotting showing EMT related gene expression in the indicated cells.

Table III. Pearson correlation analyses of the mRNA expression of SPOP and EMT related genes in human RCC samples from TCGA dataset (RNA Seq V2 RSEM).

	Pearson r	95% CI	P-value	Significant (α=0.05)	No. of samples
SPOP vs. CDH1	-0.1875	-0.27 to -0.10	<0.0001	Yes	534
SPOP vs. VIM	0.1393	0.06 to 0.22	0.0013	Yes	534
SPOP vs. ACTA2	0.185	0.10 to 0.27	<0.0001	Yes	534
SPOP vs. MMP2	0.2734	0.19 to 0.35	<0.0001	Yes	534
SPOP vs. MMP9	-0.0442	-0.13 to 0.04	0.3079	No	534
SPOP vs. TCF4	0.4277	0.36 to 0.49	<0.0001	Yes	534
SPOP vs. ZEB1	0.3942	0.32 to 0.46	<0.0001	Yes	534
SPOP vs. ZEB2	0.2884	0.21 to 0.36	<0.0001	Yes	534
SPOP vs. SMAD4	0.2086	0.13 to 0.29	<0.0001	Yes	534
SPOP vs.SNAI1	0.2581	0.18 to 0.34	<0.0001	Yes	534
SPOP vs. SNAI2	0.3063	0.23 to 0.38	<0.0001	Yes	534
SPOP vs. TWIST1	0.1092	0.025 to 0.19	0.0115	Yes	534

and metastasis. We further analyzed the association of SPOP expression and tumor recurrence-free survival, and the result demonstrated that SPOP was negatively correlated with RCC recurrence-free survival (Fig. 3B), indicating SPOP as novel prognostic marker for RCC patients.

SPOP promotes the invasiveness of RCC. To confirm the biological function of SPOP in RCC invasion, *in vitro* cell line based assays were performed. Profile of SPOP expression in a series of RCC cell lines by RT-PCR showed ACHN cells with low SPOP expression, thus, it was applied for SPOP overexpression model, while A498 cells with high SPOP was applied for SPOP silencing model (Fig. 4A and B). *In vitro* Transwell invasion assays demonstrated that overexpression of SPOP promoted ACHN invasion (Fig. 4C), while silencing of SPOP by siRNA in A498 cells suppressed cell invasion (Fig. 4D). It has been well documented that epithelial-mesenchymal transition (EMT) is a process thought to initiate metastasis, and it is characterized by the gain of mesenchymal markers (e.g., vimentin, α-SMA, MMP2) and the loss of epithelial markers (e.g., E-cadherin, cytokeratins), as well as increased motility and invasion of cancer cells (21,22).

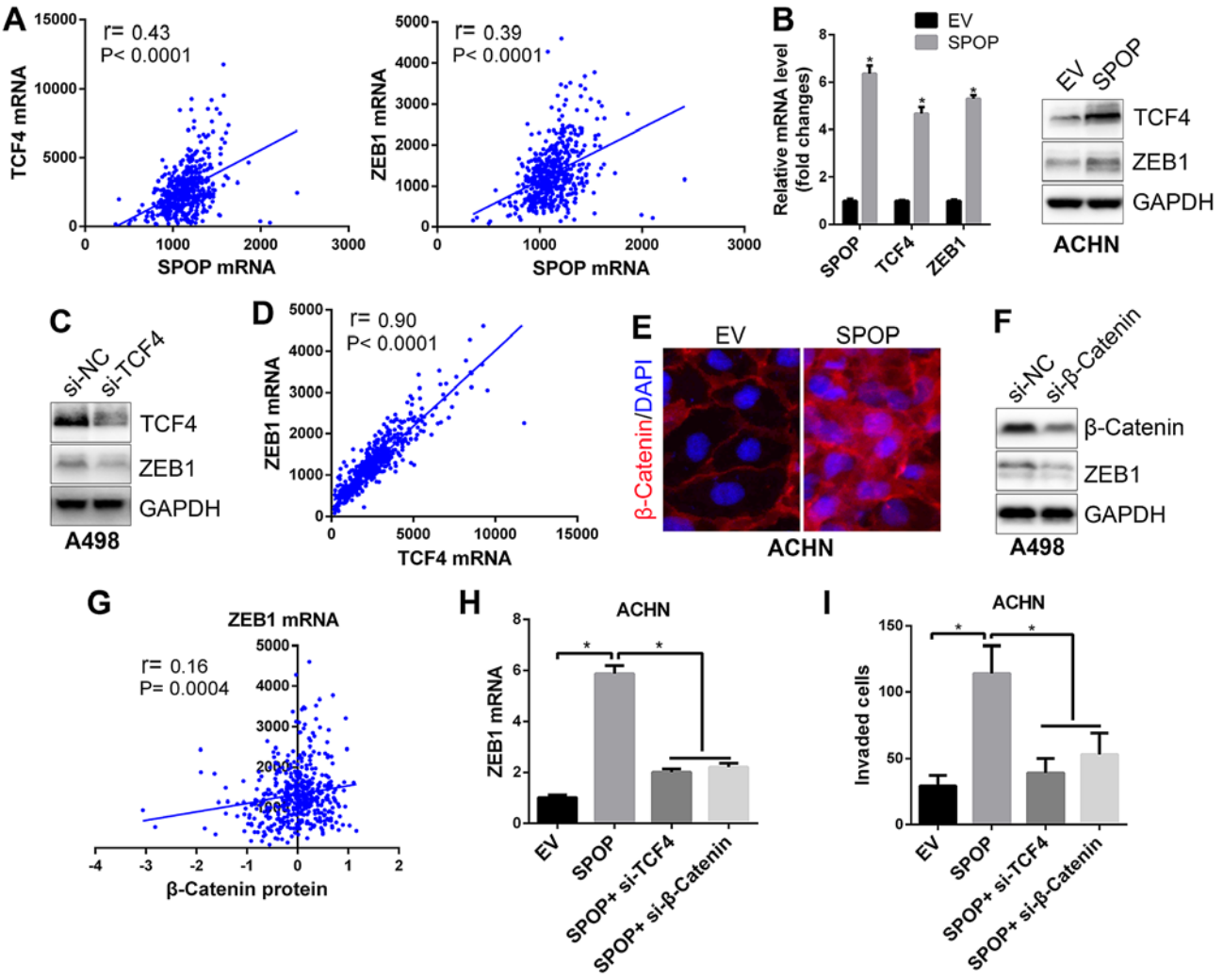

Figure 5. SPOP elevates ZEB1 expression to promote RCC cell invasion by upregulation of both β-catenin and TCF4. (A) Pearson correlation analyses of SPOP and TCF4 mRNA level, SPOP and ZEB1 mRNA level in human clear cell RCC samples from TCGA datasets. (B) RT-PCR and western blotting detecting TCF4 and ZEB1 expression in ACHN cells transfected with EV or SPOP plasmids. *P<0.05 compared to control. (C) Western blotting detecting ZEB1 expression in A498 cells transfected with control siRNA (si-NC) or TCF4 siRNA (si-TCF4). (D) Pearson correlation analysis of TCF4 and ZEB1 mRNA level in human clear cell RCC samples from TCGA datasets. (E) Immunofluorescence showing β-catenin protein expression and localization in ACHN cells transfected with EV or SPOP plasmids. (F) Western blotting detection of ZEB1 expression in A498 cells transfected with control siRNA (si-NC) or β-catenin siRNA (si-β-catenin). (G) Pearson correlation analysis of β-catenin protein and ZEB1 mRNA level in human clear cell RCC samples from TCGA datasets. (H) ACHN cells were transfected with EV, SPOP plasmids, or co-transfected with SPOP and si-TCF or si-β-catenin, then RT-PCR was performed to determine the changes of ZEB1 expression in cells. *P<0.05 between two groups. (I) Transwell assays of cell invasion of ACHN transfected with the indicated plasmids or siRNAs. *P<0.05 between two groups.

We examined the expression of EMT markers in SPOP overexpressing or silencing cells, and the results showed that SPOP downregulated epithelial markers, such as E-cadherin and Pan-CK, and upregulated mesenchymal makers, such as vimentin, α-SMA and MMP-2 (Fig. 4E). These data indicate SPOP as an inducer for the invasiveness of RCC cells.

Mechanisms of SPOP in regulation of RCC invasion. We further dissected the molecular mechanisms of SPOP in regulation of RCC invasion. Firstly, the associations between SPOP and a panel of EMT markers and EMT-inducing transcription factors (EMT-TFs) were analyzed in human clear cell RCC samples from TCGA datasets by Pearson correlation analyses. The results showed negative correlations between SPOP and epithelial marker CDH1 (E-cadherin), and positive correlations between SPOP and mesenchymal makers VIM (vimentin), ACTA2 (α-SMA) and MMP-2 (Table III). Importantly, SPOP was positively correlated with many critical EMT-TFs, such as TCF4 and ZEB1 (Table III and Fig. 5A). Further cell line based assays confirmed that overexpression of SPOP upregulated TCF4 and ZEB1 expression (Fig. 5B). ZEB1 is well known as one of the most critical transcriptional factors driving EMT in many cancer cells (23), and β-catenin/TCF4 complex has been demonstrated to bind ZEB1 gene promoter region and promote its transcription (24). Our data showed that silencing of TCF4 in RCC cells suppressed ZEB1 expression (Fig. 5C), and there was an extremely positive correlation between TCF4 and ZEB1 mRNA in clear cell RCC samples (Fig. 5D), these data again confirmed that TCF4 is an upstream regulator for ZEB1 expression. Additionally, we observed that SPOP could also upregulate cytosolic β-catenin protein expression and promote its nuclear translocation (Fig. 5E). Silencing

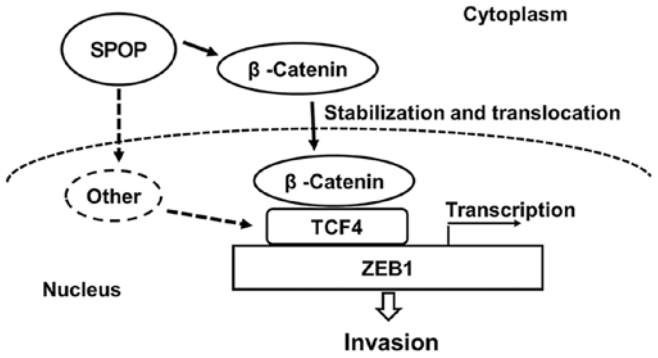

Figure 6. Putative mechanisms of how SPOP regulates ZEB1 and RCC cell invasion. SPOP may posttranscriptionally modify β-catenin protein directly or indirectly to promote its cytosolic stabilization and nuclear translocation. Thus, SPOP is able to enhance TCF4 gene expression also in a different manner, which needs further elucidation. The β-catenin/TCF4 complex is known to bind the ZEB1 promoter and activate its transcription, thus promoting tumor invasiveness.

of β-catenin suppressed ZEB1 expression (Fig. 5F), and there was a positive correlation between β-catenin protein and ZEB1 expression in clear cell RCC samples (Fig. 5G), suggesting β-catenin as upstream regulator for ZEB1. Furthermore, although overexpression of SPOP upregulated ZEB1 expression as well as cell invasion, co-transfection of TCF4 siRNA or β-catenin siRNA could ablate the effects of SPOP on ZEB1 gene expression and cell invasive ability (Fig. 5H and I). Taken together, our results indicate that SPOP promotes ZEB1 to drive RCC cell invasion via activating the β-catenin/TCF4 complex (Fig. 6).

Discussion

RCC is a clinicopathologically heterogeneous disease with distinct histological subtypes, including clear cell which accounts for the 70% of cases, and other rare subtypes, such as papillary, chromophobe, and oncocytoma (25). Although different subtypes of RCC exhibit certain distinguishing morphology, diagnostic difficulties arise when one subtype displays morphologic features that overlap with others. Recent advances are paving the way for seeking specific molecular abnormalities based on improved knowledge of the cytogenetics and molecules to recognize distinct molecular subtypes. A panel of immunohistochemical markers are used to differentiate the major subtypes of RCC. Unfortunately, these markers lack specificity and sensitivity. For example, carbonic anhydrase IX has been proposed as a sensitive marker for clear cell RCC, but it is not positive for all cases (26). Vimentin, a broad mesenchymal marker, is expressed in 87-100% clear cell and papillary RCC, but also in 73% oncocytoma (27). PAX2 is found to be a good marker for kidney cancers, but it is also positive for normal kidney tissues (28). We find that SPOP is negative in prostate cancer and bladder cancer but positive in 83% of the clear cell RCC, and all the cases with local invasion included for this study are positive. Although a large cohort of samples is required for further study, results from small number of samples in this study indicate SPOP may serve as a new marker for clear cell RCC, especial for metastatic cases.

Epithelial-mesenchymal transition (EMT) is a process thought to initiate metastasis, and it is characterized by the gain of mesenchymal markers (e.g., vimentin, α-SMA, MMP2) and the loss of epithelial markers (e.g., E-cadherin, cytokeratins), as well as increased motility and invasion of cancer cells. EMT is driven by many EMT-inducing transcription factors (EMT-TFs) (21,23). The well documented EMT-TFs include the Zinc-finger factors Snail, Slug, ZEB2 and ZEB1, and the HLH factors Twist and E12/E47. All of which directly bind to E-boxes in the promoter of the E-cadherin gene and repress its expression (29). By analyses of the associations of SPOP and EMT markers and a panel of EMT-TFs in a large cohort of clear cell RCC samples, we find SPOP is negatively correlated with epithelial maker and positively correlated with mesenchymal markers and all the EMT-TFs, suggesting SPOP indeed plays essential roles in inducing EMT and promoting RCC progression, which is consistent with the finding that SPOP predicts a poor recurrence-free survival of RCC patient.

Within the SPOP associated EMT-TFs, we further confirm ZEB1 is the critical downstream effector of SPOP to drive RCC cell invasion. ZEB1 has been reported to be regulated by the TGF-β signaling pathway (30) and Wnt/β-catenin signaling pathway (24). The activation of Wnt signaling inactivates the glycogen synthase kinase-3β, and leads to the stabilization of β-catenin protein in cytoplasm followed by the nuclear translocation to complex with TCF4 and enhance the transcriptional activity of TCF4 (31). β-catenin/TCF4 complex has been demonstrated to bind the ZEB1 promoter region and promote its transcription in intestinal tumor cells (24). We confirm that SPOP upregulates ZEB1 in clear cell RCC by promoting β-catenin protein nuclear translocation and TCF4 mRNA expression. SPOP, as an E3 ubiquitin ligase component, and appears to promote RCC tumorigenesis by the ubiquitination and degradation of PTEN, DUSP7 and Daxx as previously reported (15). However, results in this study indicate new actions of SPOP in RCC, of which SPOP seems to regulate β-catenin in posttranscriptional level and TCF4 in transcriptional level, further studies are required to illuminate the details of the mechanism.

Acknowledgements

This study was supported by the Shaanxi Provincial Key Scientific Foundation (grant no. 2013KTCL03-04 to Y. Xu), the National Natural Science Foundation of China (grant no. NSFC81172436 to Y. Sun) and the Shaanxi Provincial Natural Science Foundation (grant no. 2016JQ8011 to J. Zhou).

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