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Original Articles

YAP1-mediated pancreatic stellate cell activation inhibits pancreatic cancer cell proliferation

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ABSTRACT

Pancreatic stellate cells (PSCs) are activated in pancreatic ductal adenocarcinoma (PDAC) and are responsible for dense desmoplastic stroma. Yes-associated protein 1 (YAP1) can induce cancer-associated fibroblast activation in liver and breast tumors, but its effect on PSCs is unknown. In the present study, we determined that YAP1 was highly expressed in the nuclei of PDAC-derived activated PSCs. RNAi-mediated or pharmacological inhibition of YAP1 led to PSC deactivation. In addition, YAP1 stimulated the expression of secreted protein acidic and cysteine rich (SPARC) in PSCs, which was inhibited by RUNX1. SPARC secreted from PSCs inhibited pancreatic cancer cell (PCC) proliferation. High expression of nuclear YAP1 in tumor stroma was significantly correlated with SPARC expression and fibrosis degree in human PDAC tissues. Our study revealed a critical role for YAP1 in the regulation of PSC activation and paracrine signaling. Our findings provide insights into a novel rationale for targeting YAP1 to reprogram the PDAC microenvironment.

1. Introduction

Pancreatic cancers are the fourth-leading cause of cancer-related deaths with a 5-year survival of 8% [1]. Traditional chemotherapy has only a modest effect on the outcomes of patients with pancreatic ductal adenocarcinoma (PDAC) [2]. Moreover, until now, no promising targeted therapy is applicable for PDAC treatment. Exploration of new therapeutic strategies is urgently needed. To explore effective therapeutic targets, recent researches have focused on not only tumor cells but also the tumor microenvironment [3–6].

As reported previously by other groups and our laboratory, PDAC is histologically characterized by dense desmoplastic stroma, which builds a physical barrier to prevent drug delivery [7–9]. The pancreatic stellate cells (PSCs), also known as cancer-associated fibroblasts (CAFs), is a critical player in the formation of desmoplastic stroma by producing excessive amounts of extracellular matrix (ECM), and interacts with pancreatic cancer cells (PCCs) to regulate the oncogenesis and progression of PDAC [10]. Our laboratory and others have previously demonstrated that PSCs can promote tumor proliferation, migration, chemoresistance, and immune-evasion, and decrease the PCC dependence on glucose [11–17]. However, most preclinical studies on PSC-targeted therapies have failed, raising questions about the role of PSCs in PDAC [18].

Yes-associated protein 1 (YAP1) is a core effector of the Hippo pathway and functions as a transcription factor co-stimulatory molecule in the nucleus. When the Hippo pathway is activated, YAP1 is phosphorylated and sequestered by 14-3-3 protein in the cytoplasm, followed by degradation, which results in decreased translocation to the nucleus [19]. Previous studies demonstrated that YAP1 regulates the activation of CAFs in breast and liver cancers [20,21]. However, CAFs in different organs show distinct transcriptional fingerprints, which indicates that the biological functions of YAP1 in stromal cells may be quite different [22]. A recent study showed that inhibition of PSC activity decreased the accumulation of YAP1 in the nucleus [23]. These findings suggest involvement of YAP1 in the regulation of PSC activation, which is yet to be identified. Furthermore, secreted protein acidic

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Abbreviations

α-SMA	Alpha-smooth muscle actin
ATRA	All-transretinoic acid
CAFs	Cancer-associated fibroblasts
CHIP	Chromatin immunoprecipitation
Co-IP	Co-immunoprecipitation
CTGF	Connective tissue growth factor
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ETV1	E26 transformation-specific sequence variant 1
FAP	Fibroblast-activated protein
FGFR	Fibroblast growth factor receptor
HSCs	Hepatic stellate cells
IHC	Immunohistochemistry
PCCs	Pancreatic cancer cells
PDAC	Pancreatic ductal adenocarcinoma
PI	Propidium iodide
PSCs	Pancreatic stellate cells
RUNX1	Runt related transcription factor 1
SPARC	Secreted protein acidic and cysteine rich
STAT3	Signal transducer and activator of transcription 3
TNM	Tumor-node-metastasis
TP53INP1	TP53-induced nuclear protein 1
VP	Verteporfin
YAP1	Yes-associated protein 1

and cysteine rich (SPARC) is a matricellular glycoprotein involved in ECM assembly and cell-matrix communication during tissue remodeling, embryonic development, and tumor progression [24]. In PDAC, SPARC is mainly expressed in intra-tumor stromal cells [25]. Also, the high expression level of SPARC in stromal cells indicates poor prognosis of PDAC patients [26], especially for primary PDAC patients and those who have received gemcitabine-based chemotherapy [27]. It is speculated that SPARC expression may be related to PSC activation and tumor progression, which needs further investigation.

In the present study, we show that YAP1 knockdown inhibits both biomarkers and biological functions associated with PSC activation. Additionally, YAP1 in PSCs stimulates SPARC expression and mediates PSC-PCC interaction. We show the potential role of YAP1 in targeting PSCs to influence their activation and paracrine signaling.

2. Materials and methods

2.1. Cell culture

We isolated human PSCs from residual surgical specimens using the outgrowth method as previously reported [14,15]. Details regarding the PSC donors are displayed in Table S1. All specimens were obtained from Peking Union Medical College Hospital, according to the policies and practices of the Institutional Review Board. The human PCC line MiaPaCa-2 was purchased from ATCC (Rockville, MD, USA).

2.2. Cell transfection

YAP1-targeting siRNA (si-YAP1), SPARC-targeting siRNA (si-SPARC), and negative-control siRNA (si-NC) were designed and synthesized by Invitrogen, Carlsbad, CA, USA. The sequences are shown in Table S2. Overexpression plasmid pCMV6-SPARC and its control vector were purchased from OriGene, Rockville, MD, USA. Overexpression plasmid pcDNA 3.1-RUNX1, and its control vector, luciferase plasmid pGL3-SPARC, and Renilla plasmid pRL-TK were obtained from Shanghai GenePharma Co., Ltd., Shanghai, China. Lentivirus 358-YAP1 and control vectors were purchased from Shanghai Genechem Co., Ltd., Shanghai, China.

The si-RNAs and plasmids were transfected using lipofectamine 3000 (Invitrogen) as suggested by the manufacturer. Lentivirus was transfected using 1×10^8 TU/ml GV358 lentivirus and 5 μg/ml polybrene.

2.3. Immunofluorescence

PSCs were fixed and incubated with anti-a-SMA antibody (cat no. ab7817, Abcam, Cambridge, ENG) or anti-YAP1 antibody (cat no. 14074, Cell Signaling Technology, Inc. Beverly, MA, USA) overnight at 4 °C, followed using incubation with FITC or TRTIC-labeled secondary antibody at 37 °C for 30 min. Slides were mounted using mounting medium with DAPI, and were observed by fluorescence microscopy (IX71; Olympus, Tokyo, Japan).

2.4. Western blotting

Protein lysates were separated and transferred onto PVDF membranes. The membranes were incubated at 4 °C overnight with antibodies (more details are included in supplemental data), followed by an HRP-conjugated secondary antibody at 25 °C for 1 h. Proteins were detected using a ChemiDoc Touch System. GAPDH was used as a loading control for total protein. β-Tubulin and lamin A/C were used as loading controls for cytoplasmic and nuclear proteins, respectively.

2.5. Quantitative polymerase chain reaction (qPCR)

Each qPCR reaction was performed with power SYBR Green PCR master mix. GAPDH was used as the control. The PCR primers are listed in Table S3. In addition, the RT2 Profiler™ qPCR Array (Qiagen, Hilden, Germany) was used for identifying the upregulation or downregulation of 96 human ECM and adhesion molecule genes according to the manufacturer's protocol.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Levels of secreted SPARC in the supernatants were analyzed using a human SPARC ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

2.7. Cell counting Kit-8 (CCK-8) assay, flow cytometric assay, and collagen gel contraction

These assays are described in detail in the supplemental data.

2.8. Co-immunoprecipitation (Co-IP)

Co-IP assays were performed with a classic magnetic IP/Co-IP kit (cat no. 88804, Pierce, MA, USA). Cell lysates were incubated with anti-YAP1 antibody (cat no. sc-101199, Santa Cruz Biotechnology, California, USA) or anti-RUNX1 antibody (cat no. ab23980, Abcam) at 4 °C overnight, followed by incubation with protein A/G magnetic beads at RT for 1 h. The immunoprecipitated proteins were eluted and prepared for western-blotting analysis.

2.9. Chromatin immunoprecipitation (CHIP)

CHIP assays were performed as described previously [28]. Cellular proteins and chromatin were crosslinked and incubated with anti-RUNX1 antibody at 4 °C overnight and protein-A/G beads (cat no. 26162, Pierce) at RT for 1 h. The precipitated DNA was purified, reverse crosslinked, and prepared for PCR and qPCR.

2.10. Luciferase reporter assay

After co-transfecting HEK-293 cells with pGL3-SPARC, pRL-TK, si-YAP1, lentivirus 358-YAP1, and pcDNA3.1-RUNX1 for 48 h, cell luciferase activity measurements were performed using a dual-luciferase reporter assay system (cat no. E1910, Promega, Madison, WI, USA) according to the manufacturer's protocol.

2.11. Human PDAC tissues

One hundred and two tissue samples (73 tumor tissue samples and 29 para-tumor tissue samples) from 73 patients with PDAC from 2010 to 2014 and their corresponding clinical data were collected from Peking Union Medical College Hospital. No patient in the study received chemotherapy before surgery. Patients were staged according to the eighth tumor-node-metastasis (TNM) system [29]. This study was approved by the Institutional Review Board of Peking Union Medical College Hospital.

2.12. Immunohistochemical (IHC) staining and histological evaluation

Slides of tissue microarrays were incubated with anti-YAP1 antibody and anti-SPARC antibody. Labeled cells were incubated with HRP-conjugated secondary antibody. Staining was performed using a DAB kit (Dako, Glostrup, Denmark). YAP1 was evaluated for stromal nuclear staining while SPARC was evaluated for stromal cytoplasmic staining. Blinded analysis of all slides using the H-score method was performed independently by experienced pathologists (W.H. and Z.H.) [30], and the results were averaged. Patients were divided into two groups (high- or low- expression levels of YAP1 and SPARC) with a median H-score. The fibrosis degree was evaluated by the fibrotic change in PDAC (0%–100%) and categorized as mild-moderate fibrosis ($\leq$30%) and severe fibrosis ($>$ 30%), as previously reported [31].

2.13. Statistical analysis

Statistical analyses were performed using SPSS software, version 21.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad software Inc., San Diego, CA, USA). The means ± SD in graphs were generated from at least three repeated biological experiments by independent-sample t-testing. The associations between IHC staining and categorical variables were analyzed by Chi-square testing. Univariate and multivariate analysis of risk factors for overall survival was obtained using the Cox proportional hazard model. A two-sided $P < 0.05$ was considered to determine statistical significance.

2.14. Key resource table

Resource	Source	Identifier
Antibodies		
anti-a-SMA	Abcam, Cambridge, ENG	cat no. ab7817
anti-collagen I	Abcam, Cambridge, ENG	cat no, ab6308
anti-RUNX1	Cell Signaling Technology, Inc. Beverly, MA, USA	cat no. #4334
anti-SPARC	Abcam, Cambridge, ENG	cat no. ab89739
anti-YAP1	Cell Signaling Technology, Inc. Beverly, MA, USA	cat no. 14074
HRP-conjugated secondary Antibodies	Cell Signaling Technology, Inc. Beverly, MA, USA	cat no. #7074
anti-a-SMA	Abcam, Cambridge, ENG	cat no. ab7817
anti-collagen I	Abcam, Cambridge, ENG	cat no, ab6308
anti-RUNX1	Cell Signaling Technology, Inc. Beverly, MA, USA	cat no. #4334
anti-SPARC	Abcam, Cambridge, ENG	cat no. ab89739
anti-YAP1	Cell Signaling Technology, Inc. Beverly, MA, USA	cat no. 14074
HRP-conjugated secondary CellLine	Cell Signaling Technology, Inc. Beverly, MA, USA	cat no. #7074
HEK-3	National infrastructure of cell line resourse	cat no. 3111C0001CCC000010
MiaPaCa-2 Chemical	ATCC, Rockville, MD, USA	cat no. CRL-1420
FITC	Invitrogen, Carlsbad, CA, USA	cat no. 62-6511
lipofectamine	Invitrogen, Carlsbad, CA, USA	cat no. L3000015
SPARC ELISA kit	R&D Systems, Minneapolis, MN	cat no. DSP00
TRTIC ProteinPeptide	Invitrogen, Carlsbad, CA, USA	cat no. A16101
luciferase	Promega, Madison, WI, USA	cat no. E1910
polymerase	Applied Biosystems, Foster City, CA, USA	cat no. 4367659

3. Results

3.1. All-transretinoic acid (ATRA)-induced PSC deactivation and downregulated nuclear YAP1 expression in PSCs

PDAC-isolated PSCs appeared as spindle-shaped cells with long cytoplasmic extensions (Fig. S1A). They were positive for α-SMA and negative for oil red O with an activated phenotype [32] (Figs. S1B–C). As we previously reported [14], ATRA treatment could deactivate PDAC-isolated PSCs, which exhibited a flattened triangular morphology with increased oil-red O staining and decreased α-SMA expression (Figs. S1A–C). In addition, ATRA treatment decreased YAP1 expression in the nucleus and increased that in the cytoplasm of PSCs (Fig. S1D). The inhibition of nuclear YAP1 expression by ATRA in PSCs was further confirmed by immunofluorescence staining (Fig. S1E). These results suggest that ATRA-induced PSC deactivation, accompanied by nuclear downregulation of YAP1 in PSCs.

3.2. YAP1 inhibition led to PSC deactivation

Given that decreased nuclear YAP1 expression accompanied PSC deactivation, we speculated that YAP1 inhibition might lead to PSC deactivation. Thus, si-YAP1 transfection was conducted to knock down YAP1 in both the nucleus and the cytoplasmic protein level of PSCs, as well as the mRNA level (Fig. 1A–B). As previously reported, the connective tissue growth factor (CTGF) is a well-known target gene of YAP1 [33,34]. We found a decreased CTGF mRNA level in si-YAP1 transfected PSCs, which further suggested that YAP1 was blocked (Fig. 1C).

We next investigated whether YAP1 blockade could affect cell phenotype by detections of morphological and activation markers. We found that si-YAP1 transfection induced a transformation of the star-like shape of PSCs into a triangle or rounded outline over five days (Fig. 1D) and significantly decreased the mRNA and protein expressions of PSC activation biomarkers (α-SMA and collagen I) (Fig. 1E–F). Moreover, the drug verteporfin (VP) reportedly inhibits YAP1 by sequestering phosphorylated YAP1 in the cytoplasm, resulting in the decrease in total and nuclear protein levels of YAP1 [35,36]. We verified that VP blocked YAP1 with a decreased CTGF mRNA level and a decreased total YAP1 protein level (Fig. 1H–I). Similar to the results induced by si-YAP1 transfection, we observed a decrease in both the mRNA and protein levels of α-SMA and collagen I in a concentration-dependent manner by treatment with VP in PSCs (Fig. 1H–I).

The role of YAP1 on PSC activation was further investigated by in-vitro functional analysis. The CCK-8 assay demonstrated that PSC proliferation was significantly inhibited at 72 h after si-YAP1 transfection (Fig. 1J), and flow cytometry analysis further showed that treatment of PSCs with si-YAP resulted in a significantly higher number of cells in the G2 phase than observed for the controls (Fig. 1K). Flow cytometry analysis also revealed that the apoptotic rate of PSCs was significantly increased by YAP1-knockdown (Figure 1L). In addition, si-YAP1 transfection significantly inhibited PSC collagen gel contraction

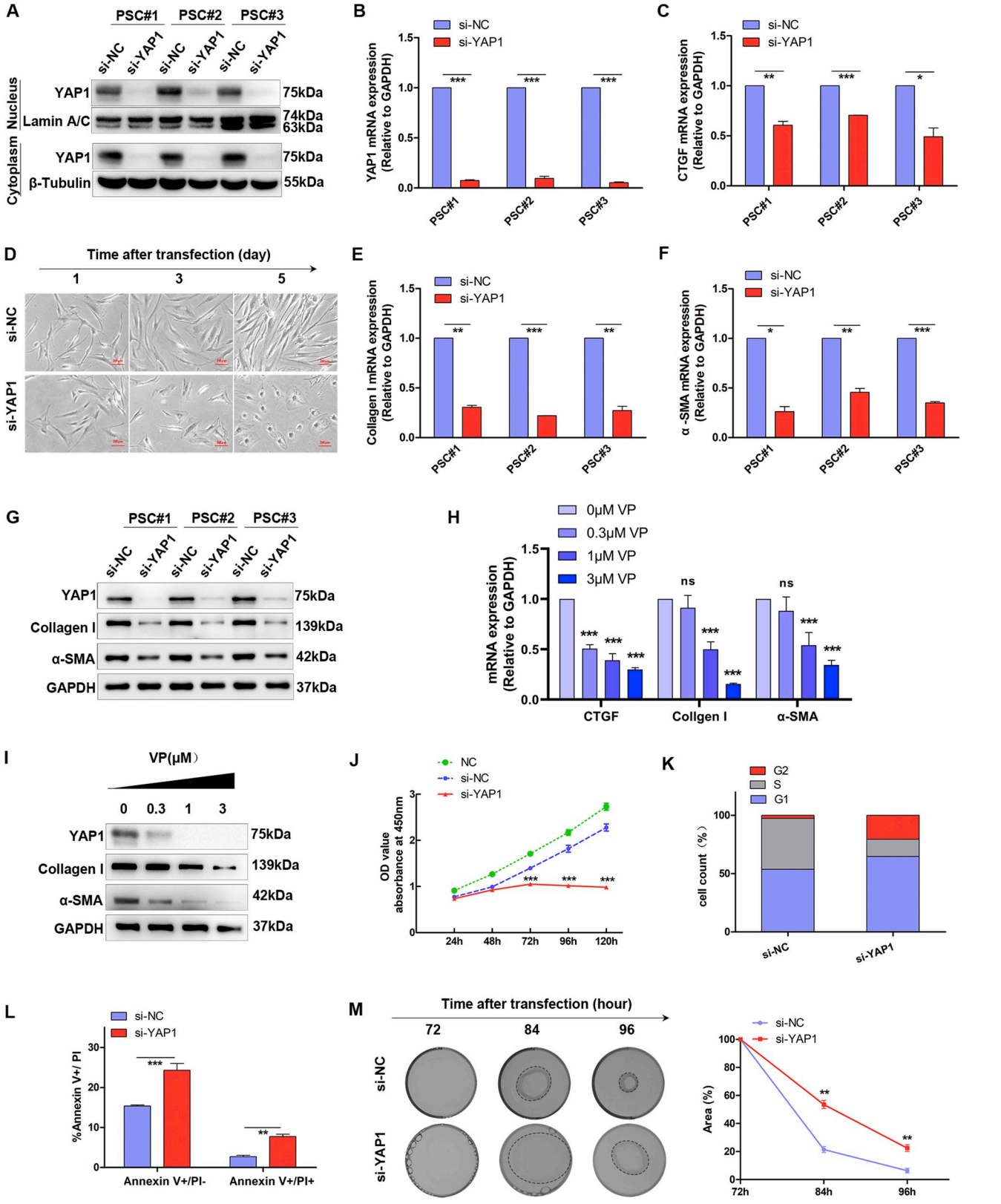

Fig. 1. YAP1 depletion leads to PSC deactivation. (**A**) YAP1 knockdown was detected by western blotting in both the nucleus and cytoplasm of si-YAP1-transfected PSCs. (**B**) YAP1 knockdown was detected by qPCR of si-YAP1-transfected PSCs. (**C**) The influence of si-YAP1 transfection on CTGF was verified by qPCR analysis. (**D**) Morphological changes were observed in PSCs at 1, 3, and 5 days after YAP1 knockdown. (**E-F**) qPCR analysis showed RNA expression of α-SMA and collagen I affected by YAP1 knockdown. (**G**) Representative western blotting showed total protein levels of YAP1, α-SMA, and collagen I affected by YAP1 knockdown. (**H**) PSCs were exposed to 0, 0.3, 1, and 3 μM VP in serum-free DMEM/F12 for 2 days away from light. The influence of VP on the expressions of CTGF, α-SMA, and collagen I was detected by qPCR. (**I**) The influence of VP on expressions of YAP1, α-SMA, and collagen I was investigated using western blotting. (**J**) Cell proliferation was measured daily from 24 h to 120 h using CCK-8 assays. (**K**) The influence of YAP1 knockdown on cell cycle arrest was analyzed by flow cytometry. (**L**) The influence of YAP1 knockdown on apoptosis rate was analyzed by flow cytometry. (**M**) Changes in collagen contractility were observed at 72, 84, and 96 h after YAP1 knockdown. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

(Figure 1M). All these results suggest that YAP1 inhibition leads to PSC deactivation.

3.3. YAP1 upregulated SPARC expression in PSCs

To investigate the impact of YAP1 on ECM derived from PSCs, mRNA expression in these cells was analyzed using a human extracellular matrix and adhesion molecules PCR array. Thirty-one genes were identified with greater than 5-fold change in YAP1-knockdown PSCs, compared with controls (Table S4). Of these, 30 genes were identified as downregulated and 1 as upregulated.

Notably, SPARC was among the most differentially expressed ECM proteins, with a 13.33-fold expression decrease in YAP1-knockdown PSCs. We further verified the effects of YAP1 on SPARC production in three separate preparations of primary human PSCs. YAP1 knockdown significantly decreased mRNA and protein expression and secretion of SPARC in all three PSCs (Fig. 2A–C). As expected, YAP1 overexpression significantly increased SPARC expression and secretion (Fig. 2D–F). Therefore, our results indicated that YAP1 could upregulate the expression of SPARC in PSCs.

3.4. YAP1-induced SPARC expression was suppressed by runt related transcription factor 1 (RUNX1)

We retrieved YAP1-interacting proteins with a 0.7 threshold score using the Mentha database (http://mentha.uniroma2.it). The predicted proteins that interacted with YAP1 are shown in Fig. 3A. Among these, RUNX1 was predicted to bind to the SPARC DNA promoter at three binding sites by the Jaspar database (http://jaspar.genereg.net/), an open-access database for eukaryotic transcription factor binding profiles (Fig. 3B). Co-IP demonstrated a physical interaction between YAP1 and RUNX1 in PSCs (Fig. 3C–D). The CHIP assay confirmed the binding of the RUNX1 protein to putative binding sites of the SPARC promoter in PSCs (Fig. 3E–F).

To better understand the role played by YAP1 in the regulation of SPARC, the luciferase reporter assay was performed in HEK-293 cells. YAP1 knockdown significantly decreased SPARC luciferase activity, and YAP1 overexpression significantly increased SPARC luciferase activity in comparison with controls (Fig. 3G). Moreover, we observed that YAP1 overexpression had no effect on SPARC luciferase activity when RUNX1 was overexpressed (Fig. 3G). RUNX1 overexpression, with or without YAP1 knockdown, significantly decreased SPARC luciferase activity (Fig. 3G). Furthermore, we demonstrated that YAP1 knockdown upregulated RUNX1 expression in PSCs (Fig. 3H). Either YAP1 knockdown or RUNX1 overexpression could significantly downregulate SPARC expression. Simultaneous RUNX1 overexpression and YAP1 knockdown led to more significant inhibition of SPARC expression (Fig. 3H). These data suggest that YAP1 stimulates SPARC expression at the transcriptional level, which can be suppressed by RUNX1.

3.5. YAP1 mediated the impact of PSCs on PCC proliferation via paracrine SPARC

We further explored whether the paracrine roles of SPARC mediated by YAP1 affected PCC proliferation. Both si-RNA and the overexpression plasmid were used to knock down and overexpress proteins (YAP1 and SPARC) in PSCs, respectively (Fig. 4A–B). The effects of PSCs on PCCs was investigated by incubating MiaPaCa-2 with PSC-conditioned medium (Fig. 4C). YAP1-knockdown PSCs significantly increased MiaPaCa-2 proliferation, which was attenuated by SPARC overexpression in PSCs or treatment with exogenous SPARC (Fig. 4D). Accordingly, YAP1-overexpressing PSCs significantly decreased MiaPaCa-2 proliferation, which could be antagonized by SPARC knockdown in PSCs (Fig. 4E). Similar results were demonstrated by the PSC-PCC co-culture system (Figs. S2A–C). These results suggest that YAP1 in PSCs inhibits PCC proliferation through paracrine SPARC.

3.6. YAP1 and SPARC expressions in PDAC tissues

The basic characteristics of 73 patients with PDAC, including 45 men and 28 women with a median age of 61 years, are shown in Table S5. YAP1 was mainly expressed in the nuclei of tumor cells and stromal cells (Fig. 5A). SPARC was only expressed in the cytoplasm of PDAC

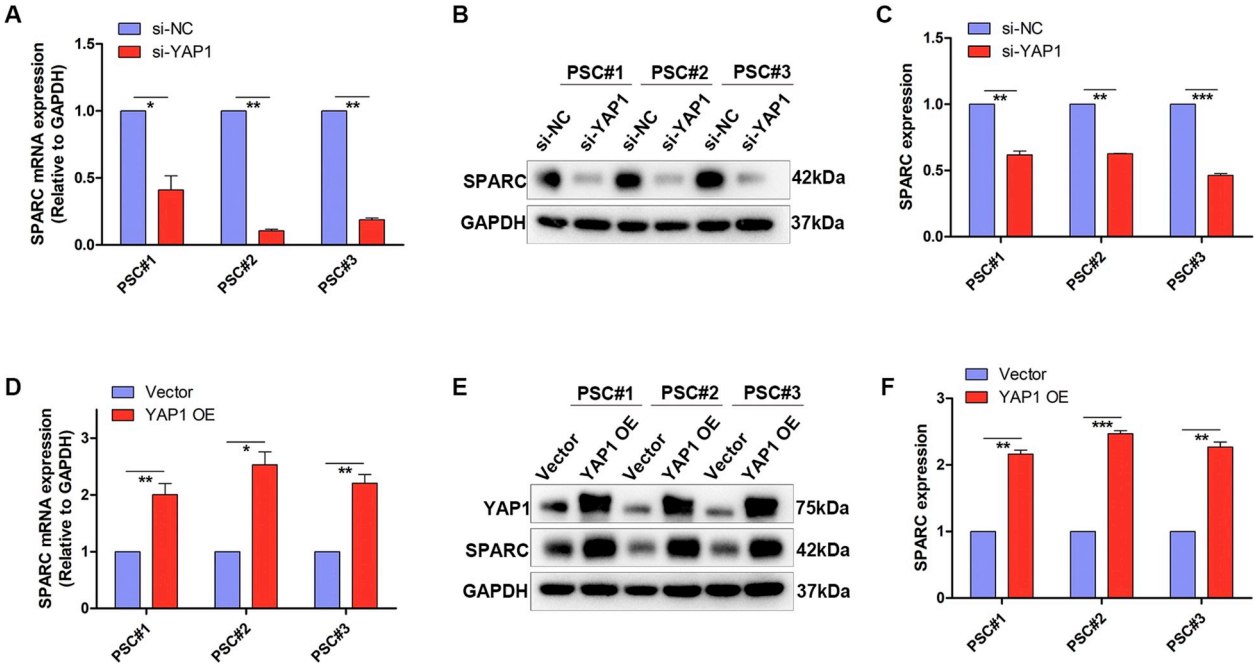

Fig. 2. YAP1 stimulates SPARC expression and secretion in PSCs. (**A-C**) Downregulation of SPARC was validated by qPCR, western blotting, and ELISA in three separate si-YAP1-transfected PSC preparations. (**D-F**) Upregulation of SPARC was validated by qPCR, western blotting, and ELISA in three separate YAP1-overexpressed PSC preparations. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

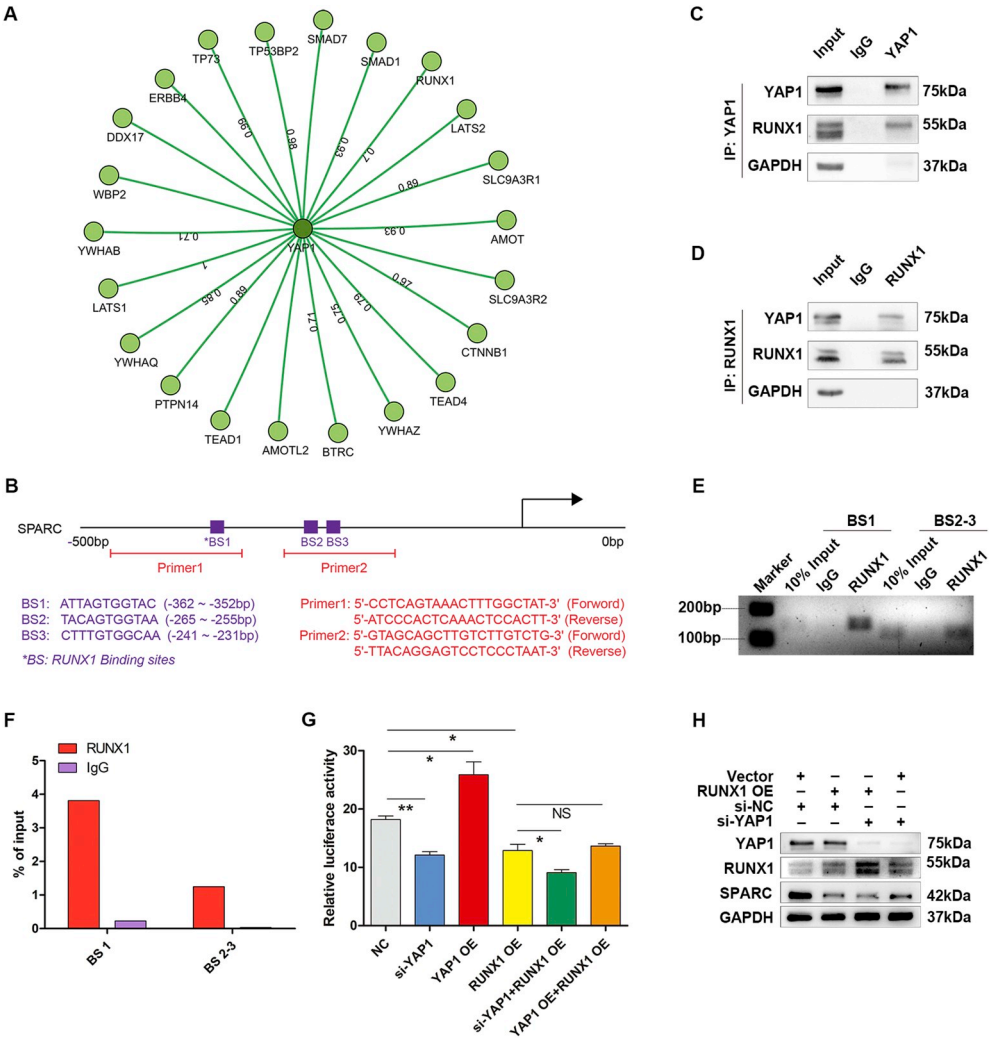

Fig. 3. YAP1 stimulates SPARC expression and is suppressed by RUNX1. (**A**) A diagram of predicted YAP1-interacting transcription factors. (**B**) The predicted SPARC promoter sequences bound by RUNX1 and their CHIP-PCR primers. (**C-D**) The interaction between YAP1 and RUNX1 in PSCs was determined by Co-IP. IgG was used as the control. (**E-F**) The binding of RUNX1 to predicted SPARC promoter binding regions was confirmed in PSCs using CHIP-PCR and CHIP-qPCR. IgG was used as the control. (**G**) SPARC transcription activity was detected in HEK-293 cells by transfection with SPARC luciferase, YAP1 and RUNX1 overexpression plasmid and si-YAP1. (**H**) Western blotting analysis showed that SPARC expression was downregulated in RUNX1-overexpressed or (and) YAP1 knockdown PSCs, and that RUNX1 expression was upregulated in si-YAP1 transfected PSCs. Only the uppermost band of RUNX1 was the RUNX1-specific band. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

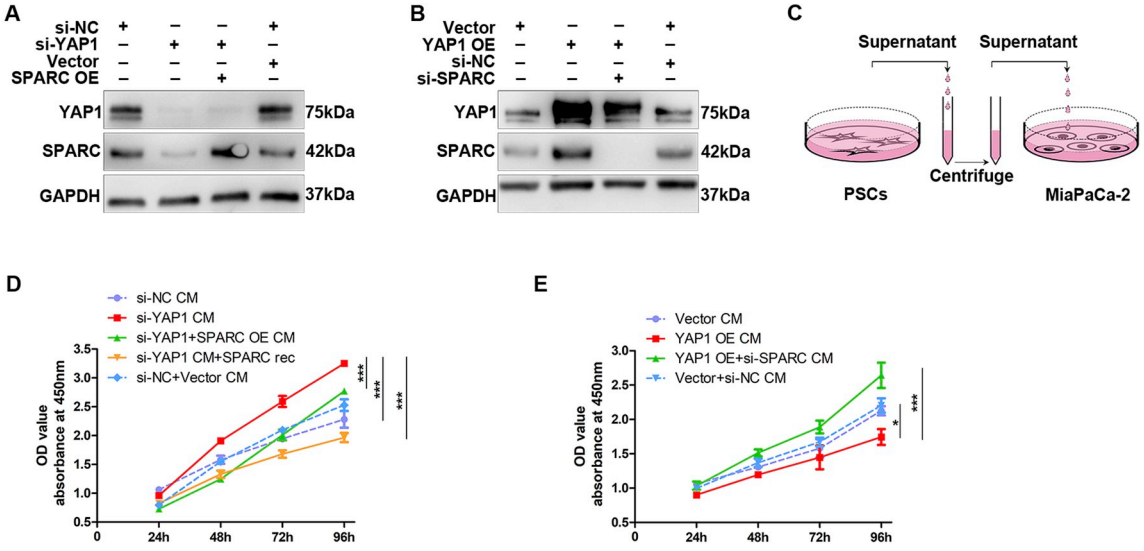

Fig. 4. YAP1-knockdown PSCs promote PCC proliferation. (**A-B**) PSCs were subjected to knockdown or overexpression of YAP1 and SPARC and confirmed using western blotting. (**C**) The incubation model of MiaPaCa-2 with PSC-conditioned medium. (**D-E**) MiaPaCa-2 proliferation was measured daily from 24 to 96 h using CCK-8 assays. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

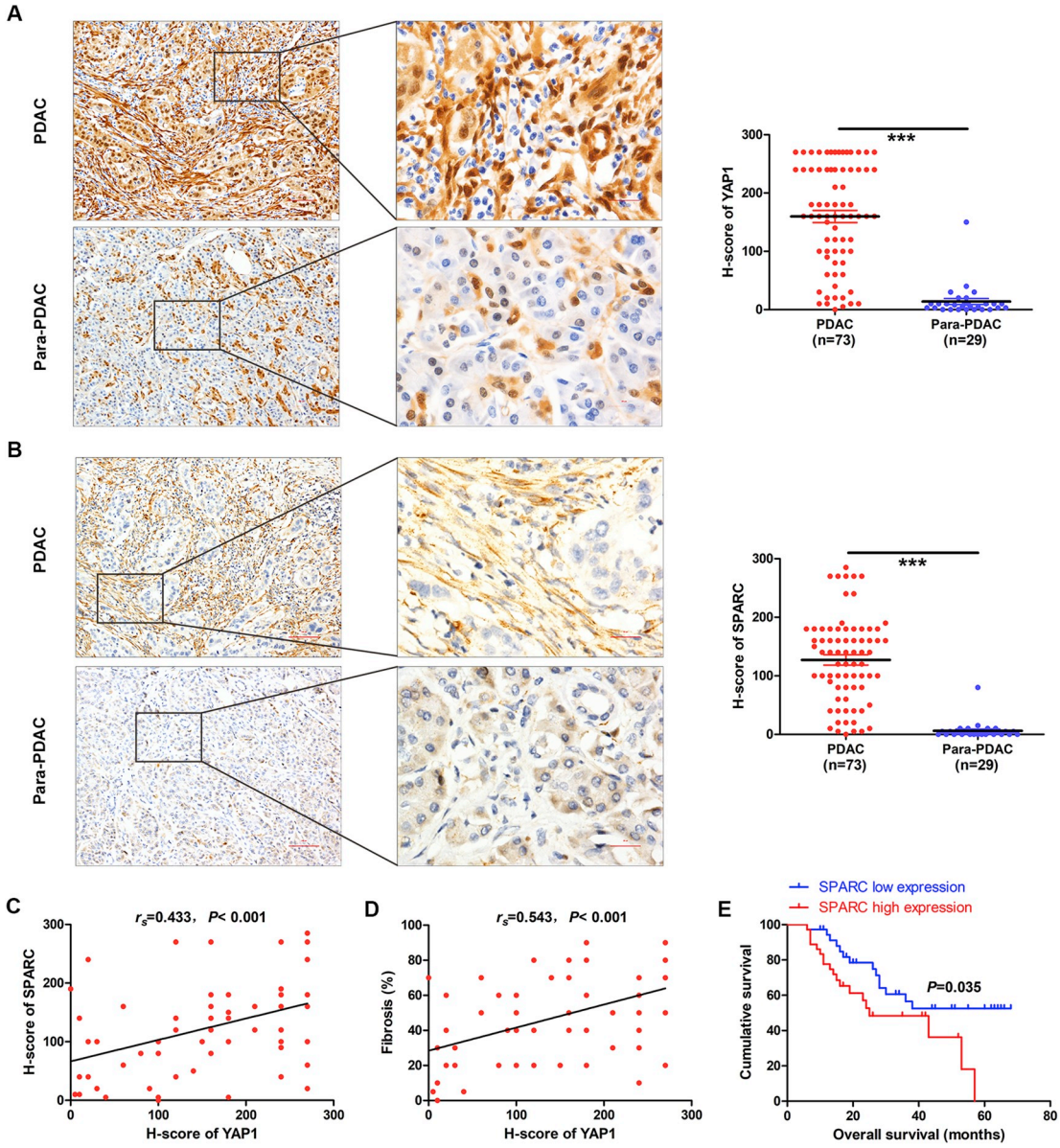

Fig. 5. YAP1 and SPARC expressions in PDAC tissues. (**A-B**) Representative figures of YAP1 and SPARC IHC as well as the H-score comparisons between 73 PDAC and 29 para-PDAC tissues (Scale bar = 100 μM, 25 μM). (**C**) H-scores of YAP1 and SPARC were significantly correlated in PDAC tissues. (**D**) YAP1 H-scores were significantly correlated with fibrosis (%). (**E**) Kaplan-Meier survival curve of SPARC expression in PDAC. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

stromal cells (Fig. 5B). The IHC assay showed that H-scores of YAP1 and SPARC were significantly higher in PDAC than in para-PDAC (Fig. 5A and B), and they had a significantly positive correlation (r = 0.433, P < 0.001) (Fig. 5C). The YAP1 H-score was significantly positively correlated with the degree of fibrosis (r = 0.543, P < 0.001) (Fig. 5D). A high SPARC H-score was significantly associated with large tumor size (P < 0.05) (Table 1). Additionally, univariate analysis showed that tumor size, lymph node metastasis, TNM-stage, tumor differentiation, and stromal SPARC expression (Fig. 5E) were prognostic factors for PDAC overall survival (P < 0.05) (Table S6). In further multivariate analysis, poor tumor differentiation and increasing stromal SPARC expression were independent prognostic factors for poor outcome in PDAC (P < 0.05) (Table S6).

4. Discussion

YAP1 is expressed at lower levels in quiescent PSCs of normal tissues than in activated PSCs of mouse early pancreatic neoplasm and human PDAC [37]. However, the detailed mechanisms of YAP1 in the regulation of PSCs have not been demonstrated before. In our study, the knockdown of YAP1 downregulated α-SMA and collagen I expression in PSCs, suppressed cell proliferation and contractility, and increased apoptosis in PSCs, suggestive of PSC phenotype switching to inactivation. Furthermore, YAP1 in PSCs promoted the expression and secretion of SPARC to prevent PCC proliferation. And the YAP1-SPARC regulation could be inhibited by RUNX-1. Our study is the first to reveal the effect and mechanism of YAP1 on activation and paracrine function of PSCs (Fig. 6).

This study not only showed a decrease in YAP1 expression upon PSC deactivation, but also found that YAP1 knockdown led to PSC deactivation. Our results agree with those of Mannaerts et al. [21], who reported that knock down of YAP1 resulted in the inability of hepatic stellate cells (HSCs) to be activated. Calvo et al. [20] found that YAP1 was necessary for the generation and maintenance of CAFs in breast tumors by regulating mechano-transduction. These results indicate the crucial role of YAP1 in driving stellate cell activation and sustaining the

Table 1
Associations between clinicopathological parameters and H-score of YAP1 and SPARC in 73 PDAC patients.

Parameter	YAP1 n(%)		*P*	SPARC n(%)		*P*
	Low	High		Low	High	
Total	37(50.7)	36(49.3)		37(50.7)	36(49.3)	
Age			0.114			0.534
< 60 years	18(24.7)	11(15.1)		16(21.9)	13(17.8)	
≥60 years	19(26.0)	25(34.2)		21(28.8)	23(31.5)	
Gender			0.291			0.067
Male	25(34.2)	20(27.4)		19(26.0)	26(35.6)	
Female	12(16.4)	16(21.9)		18(24.7)	10(13.7)	
Tumor location			0.563			0.295
Head	21(28.8)	18(24.7)		22(30.1)	17(23.3)	
Body/Tail	16(21.9)	18(24.7)		15(20.5)	19(26.0)	
Tumor size			0.114			0.040*
≤4 cm	18(24.7)	11(15.1)		19(26.0)	10(13.7)	
> 4 cm	19(26.0)	25(34.2)		18(24.7)	26(35.6)	
Lymph node metastasis			0.879			0.262
No	14(19.2)	13(17.8)		16(21.9)	11(15.1)	
Yes	23(31.5)	23(31.5)		21(28.8)	25(34.2)	
TNM-stage			0.677			0.677
I	13(17.8)	11(15.1)		13(17.8)	11(15.1)	
II-IV	24(32.9)	25(34.2)		24(32.9)	25(34.2)	
Tumor differentiation			0.854			0.854
Well/Moderate	26(35.6)	26(35.6)		26(35.6)	26(35.6)	
Poor	11(15.1)	10(13.7)		11(15.1)	10(13.7)	
Fibrosis			0.020*			0.001**
Mild/Moderate	14(19.2)	5(6.8)		16(21.9)	3(4.1)	
Severe	23(31.5)	31(42.5)		21(28.8)	33(45.2)	

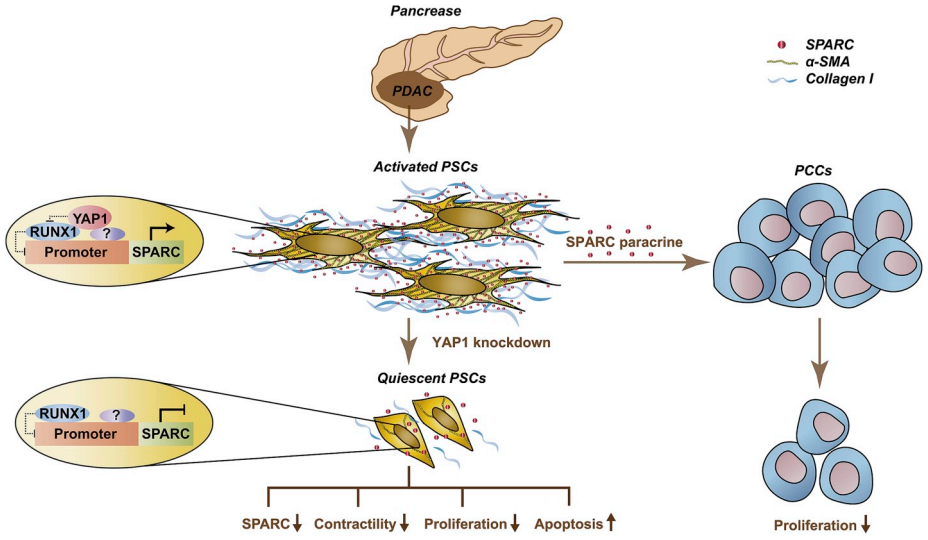

Fig. 6. Schematic diagram depicting the effect of YAP1 on PSC activation and paracrine signaling.

activated phenotype of PSCs. Additionally, activated PSCs are vital producers of desmoplastic stroma. Our IHC analysis of human tissues suggests that increasing stromal YAP1 is associated with the development of desmoplastic stroma in PDAC, which is in accordance with the results in vitro. Together, the targeting of YAP1 is a feasible strategy for inactivating PSCs and may provide novel therapeutic opportunities to inhibit desmoplastic reactions in PDAC.

Transcriptional regulators for SPARC have been explored in a variety of tumor cells, including fibroblast growth factor receptor (FGFR), signal transducer and activator of transcription 3 (STAT3), TP53-induced nuclear protein 1 (TP53INP1), and E26 transformation-specific sequence variant 1 (ETV1) [38–42], which are seldom investigated in stromal cells. Our study showed that YAP1 dramatically upregulated SPARC expression in PSCs, and nuclear expression of YAP1 in stromal cells was positively correlated with SPARC expression in tumor stroma in PDAC patient samples. Moreover, our luciferase reporter assay verified that YAP1 stimulated the transcriptional activity of the SPARC promoter. All these results suggest that SPARC is a YAP1-target gene. This speculation is supported by the finding that YAP1 can bind to the SPARC promoter in a CHIP-seq analysis [43]. In addition, we demonstrate that RUNX1, a heterodimeric transcription factor, interferes with YAP1-mediated SPARC expression. We observed that overexpression of RUNX1 inhibited the transcriptional activity of SPARC, even when YAP1 was overexpressed. These finding suggests that RUNX1 can inhibit the expression of the YAP1 target gene, identical to the results of a previous study [44]. Also, we found that YAP1 inhibited RUNX1 expression in PSCs, suggesting YAP1 itself can alleviate the repressive role of RUNX1 in SPARC transcription. It is speculated that YAP1 may stimulate RUNX1 downregulation through ubiquitination [45,46]. Therefore, YAP1, as a transcription co-factor, stimulates SPARC expression, which can be attenuated by RUNX1.

It has been demonstrated that activated pancreatic stellate cells

(PSCs) can produce large amounts of ECM, including hyaluronan, collagens, and SPARC, and play a central role in the desmoplastic reaction in PDAC. A high level of PSC activity (dependent on the expression of α-SMA, collagen I, and hyaluronan) has been reported to be related to poor outcomes of PDAC patients [47,48]. In consistent with this, Han et al. [49] demonstrated that increased SPARC expression in PDAC stromal cells was associated with a poor prognosis through a meta-analysis. Our present study also demonstrated a similar result that SPARC overexpression in PDAC stroma indicated poor outcomes. Furthermore, Guweidhi et al. [50] found that SPARC promoted tumor invasiveness by up-regulating the expression of MMP-2 in PDAC. It has been speculated that tumor invasiveness promoted by increased SPARC expression might account for poor prognosis in PDAC [51].

However, the roles of PSCs and SPARC in PDAC might be more complicated. Recent findings suggest that PSCs also have a protective effect on tumors. Depletion of α-SMA + fibroblasts in vivo results in accelerated tumor growth and worsened prognosis [52–54]. High levels of PSC activity, which is defined by increasing expression of fibroblast-activated protein (FAP), is related to increased survival of PDAC patients [55]. The contradictory results of different studies on PSCs may be due to three reasons, as Sun et al. [18] suggested. Firstly, the heterogeneous characteristics of PSCs with diverse sources and functions are associated with different bio-markers and secreted proteins. Recent reports regarding the heterogeneity of PSCs have been summarized by Awaji et al. [56]. Experimental designs highlighting different approaches to PSCs results in a biphasic effect on PDAC. Secondly, PSCs may differentiate into diverse subpopulations with specific functions among different patients. Thirdly, the differentiation and function of PSCs are dynamic during the development of PDAC. Unfortunately, the present study as well as the studies mentioned above did not classify and analyze PSCs based on their heterogeneity and differentiation, which needs to be taken into consideration in future research.

In addition to PSCs, SPARC might have a protective effect on tumors as well. As reported, the knockout of SPARC enhanced the growth of PDAC in mice [57,58]. Exogenous SPARC inhibited PCC proliferation by upregulating the expression of p21, leading to a cell cycle arrest at G1 [50], which is in consistent with our results. Interestingly, we found that high SPARC H-score was significantly associated with larger tumor size and poor outcome in human PDAC samples in PDAC. SPARC-promoted tumor infiltration into adjacent pancreatic tissue by affecting tumor-ECM interactions might partially explain the controversial result between in vitro and in vivo assays in our study [51,59]. In addition, the role of SPARC in cancer has been demonstrated to be highly dependent on the initiating cell type, tumor, stage, and the microenvironment context. The effects of SPARC on the growth and invasion of PCCs might be correlated with the interaction between SPARC and other growth factors and the hypoxic environment of PDAC in vivo [51]. The correlation between SPARC and tumor cell invasion and the interaction between SPARC and the tumor environment in PDAC need further clarification. All these results suggest that SPARC expression and PSC activation may play a complex, multifaceted role in PDAC.

In conclusion, YAP1 knockdown mediates PSC deactivation. YAP1 upregulates SPARC expression in PSCs to inhibit PCC proliferation. Our study reveals the key effect of YAP1 on PSC activation and paracrine signaling, and the controversial role of PSCs and YAP1/RUNX1/SPARC in PDAC. Our findings provide insights into a novel rationale for targeting YAP1 to reprogram the PDAC microenvironment.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Ethics approval and consent to participate

The tissue samples were collected and used in accordance with approval by the institutional review board of Peking Union Medical College Hospital.

Acknowledgments

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.07.015.

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