SPOCK1 promotes tumor growth and metastasis in human prostate cancer

Abstract

Prostate cancer is the most diagnosed non-cutaneous cancercancers and ranks as the leading cause of cancer-related deaths in American males.US men. Metastasis is the primary cause of prostate cancer mortality. Although the five-year survival rate for localized prostate cancer is nearly 100%, the one for metastatic prostate cancer is only 28%. Survival rate for metastatic patients is only 28%, while it is nearly 100% for localized prostate cancers. MolecularWhile the molecular mechanisms that underliesunderlying this malignancy remain obscure.,. theThe present study investigated the role of SPOCK1, which contains osteonectin-like domains, a Kazal-like sequence, and a cys-trp-cys-val domain, SPARC/osteonectin, cwcv and kazal-like domains proteoglycan 1 (SPOCK1) in prostate cancer the progression. of prostate cancer. Initially, weWe found that SPOCK1 expression of SPOCK1 was significantly higher in prostate cancer tissues relative tocompared with the non-cancerous tissues. In particular, the SPOCK1 expression of SPOCK1 was also markedly higher in the metastatic tissues compared with non-metastatic cancerous tissues. Knockdown and overexpression of SPOCK1 studies expression by specific shRNA in PC3 cells significantly inhibited, whereas overexpression of SPOCK1 in RWPE-1 cellshighlighted its role to promote promoted cell proliferation andviability, colony formation *in vitro*, and the tumor growth *in vivo*. Moreover, theknockdown of SPOCK1 knockdown in PC3 cells was associated with cell cycle arrest in G0/G1 phase and the SPOCK1 overexpression of SPOCK1 in RWPE-1 cells induced cell cycle arrest in S phase. Knockdown of SPOCK1 knockdown in PC3 cells alsoeven increased cell apoptosis. Modulation of SPOCKk1 modulation was also observed to affect cancerous cell proliferation and apoptotic processes in the mouse model of prostate cancer. In addition, the SPOCK1 knockdown of SPOCK1 decreased, whereas the SPOCK1while overexpression of SPOCK1 increased cell migration and invasion abilities in vitro. Injection of SPOCK1-depleted PC3 cells significantly decreased the metastatic nodules in mouse lungs. These findings altogether suggest that SPOCK1 is a critical mediator

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of tumor growth and metastasis in prostate cancer.

Keywords: SPOCK1; tumor progression~~growth~~; tumor metastasis; prostate cancer.

Introduction

Prostate cancer is the most diagnosed non-cutaneous cancer~~cancers~~ and ranks as the second leading cause of cancer-related deaths in American males~~men [1]~~. [1] Based on ~~According to a~~ recent statistics, ~~there were~~ 238,590 newly~~new~~ diagnosed cases of prostate cancer were reported;~~,~~ among these cases,~~which~~ 29,720 cases of American males were estimated to die in 2013, which makes this cancer as~~ in the US men, making it~~ the most serious health problem among male patients~~ [2]~~. [2]~~.~~ Metastasis is the primary factor~~attributor~~ for prostate cancer mortality ~~deaths of this malignancy [3]~~. [3] The ~~It is estimated that the~~ five-year survival rate for patients diagnosed with metastatic prostate cancer is estimated to be 28%; by contrast, such rate~~28%, while it~~ is nearly 100% for localized patients~~ [4]~~. [4] The~~Even~~ worse finding is that~~,~~ the overall survival has not changed in the last 20 years among~~in~~ patients who suffer~~suffering~~ from metastatic prostate cancer. However,~~ though~~ an approximately~~approximate~~ 40% decrease in the mortality of this malignancy has been achieved over the last two decades. Hence, the means~~how~~ to prevent prostate cancer progression and to perform necessary interventions~~make early interference~~ before this cancer metastasizes~~it metastasize~~ to other organs remain~~remains~~ a major clinical challenge.

SPARC/osteonectin, cwcv, and kazal-like domain~~domains~~ proteoglycan 1 (SPOCK1, also known as testican1) is a proteoglycan that belongs to a novel Ca^{2+}-binding proteoglycan family. Members of this family, which share~~shares a similar structure~~homologous domains that includes~~including~~ N-terminus, follistatin-like domain, and C-terminus, are implicated in cell proliferation, cell~~-~~-cell adhesion, and migration~~ [5]~~. [5] SPOCK1 has been observed to play crucial roles in cell cycle regulation, cell apoptosis, DNA repair, and metastasis~~ [6]~~. [6] ~~Expression of~~ SPOCK1 expression was fairly high in the brain~~ [7]~~. [7] This proteoglycan is~~It was~~ also present

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in other tissues, such as cartilagescartilage [8] and myoblasts[9]. [9] et al. More interestingly, a number ofmounting evidence studies haves shown that SPOCK1 plays critical roles in hepatocellular carcinoma progression [10] and glioblastoma invasion [11]. [11] SPOCK1 can regulate the epithelial–mesenchymal transition (EMT) process in lung cancer [12]. [12] Moreover, SPOCK1-mediated EMT signaling confers acquired resistance to lapatinib in HER2-positive gastric cancer [13]. [13] SPOCK1 can even serve as a potential prognostic marker in gallbladder cancer [14]. [14] All these studies suggest the extensive roles of SPOCK1 in human tumorigenesis.tumoriogenesis. The most noticeable finding is that SPOCK1 was first isolated in human testes, and eventuallylater, two studies reported the aberrant expression of SPOCk1 in prostate cancer [15, 16]. [15, 16] However, minimal data has shown ifbeenit is still unclear that whether showed on whether SPOCK1 plays any role in prostate tumorigenesistumoriogenesis and prostate cancer progression.

The present study aimed to investigate profilethe SPOCK1 expression profiles of SPOCK1 in prostate cancer, with a special focus on its expression in metastatic tissues. For the functional studies, specific shRNA against SPOCK1 (shSPOCK1) and its overexpression plasmid were employed. Systemic study of the effects of SPOCK1 modulation effects on tumor growth and metastasis in prostate cancer will also be totally conducted in the current studyhere.

Results

SPOCK1 is overexpressed in prostate cancer tissues.

Initially, we performed qRT-PCR analysis of the SPOCK1 mRNA levels of SPOCK1 in 20a consecutive of 20 prostate cancer cases. Our data showed that the relative mean mRNA level of SPOCK1 in the cancerous tissues was approximately two2-fold of that in the adjacent non-cancerous tissues (Figure. 1A). Moreover, we performed IHC analysis in 50 cases of prostate cancer cases. The IHC staining revealed that SPOCK1 was densely stained in the tumor tissues, whereas this proteoglycanit was rarely detected in non-tumor tissues (Figure. 1B). Further analysis showed that 32 of the 50

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cases (64%) were strongly stained with a score over 4 in the tumor samples. On the contrary, only 13 of the 50 cases (26%) were strongly stained with a score over 4 in the noncancerous samples (Figure. 1C). Interestingly, an average staining score of SPOCK1 in the 24 metastatic cases were significantly higher than that of the 26 non-metastatic cases (Figure. 1D). These observations strongly suggest the high SPOCK1 expression ~~of SPOCK1~~ in prostate cancer tissues, particularly in the metastatic tissues.

Successful modulation of SPOCK1 expression in prostate cancer cells

Furthermore, we performed ~~W~~western~~western~~ blot analysis of SPOCK1 expression in 5 prostate cancer cell lines. Our data showed a variety of~~that~~ SPOCK1 expressions in these cell lines ~~were differentially expressed~~, with its highest expression present in PC3 cells and lowest~~east~~ level in RWPE-1 cells (Figure. 2A). ~~This result~~These ~~made~~results made PC3 and RWPE-1 as our optimal cell lines for subsequent functional analyses. We employed specific shRNA to deplete SPOCK1 expression ~~of SPOCK1~~ in the PC3 cell line~~,~~ and to upregulate~~up-regulated~~ SPOCK1 in the RWPE-1 cell line with its expression plasmid. Transfection of PC3 cells with the specific shRNA against SPOCK1 (shSPOCK1) significantly decreased the SPOCK1 mRNA level ~~of SPOCK1~~ in PC3 cells (Figure. 2B), whereas~~while transfection of~~ SPOCK1 plasmid transfection into RWPE-1 cells increased its mRNA level by up to 4.5 ~~-~~folds (Figure. 2C). Consistently, the SPOCK1 protein level ~~of SPOCK1~~ was decreased in response to its specific shRNA~~,~~ and increased through~~by~~ transfection of its expression plasmid (Figure. 2D). These data confirmed the successful construction of prostate cancer cell~~s~~ lines ~~that were stably depleted~~with either stable konckdown of SPOCK1 (PC3 cells) or overexpression of~~overexpressing~~ SPOCK1 (RWPE-1 cells).

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Modulation of SPOCK1 expression affected cell proliferation in vitro~~.~~

To study the effects of SPOCK1 modulation on prostate cancer cell proliferation, we performed MTT assay to assess cell viability in PC3 cells (Figure. 3A) and RWPE-1 cells (Figure. 3B). Cell numbers were monitored for six~~in a~~ consecutive ~~of 6~~ days in both cell lines. In PC3 cells, the SPOCK1 knockdown ~~it~~ was observed to decrease~~that~~

~~knockdown of SPOCK1 decreased~~ the cell viability since day 3. By day 6, cell viability was only half of the control cells (Figure. 3A). On the contrary, the SPOCK1 overexpression ~~of SPOCK1~~ in RWPE-1 cells increased cell viability since day 3 (Figure. 3B). Moreover, we~~We also~~ performed colony formation assay (Figure. 3C). The SPOCK1 ~~It was shown that~~ knockdown was shown to~~of SPOCK1~~ significantly decrease ~~decreased the~~ colony formation in PC3 cells, whereas the SPOCK1 overexpression ~~of SPOCK1~~ markedly increased the number of colonies in RWPE-1 cells (Figure. 3D).

~~Modulation of~~ SPOCK1 ~~interrupted~~ regulated cell cycle progression and cell apoptosis process~~.~~

Cell cycle ~~progression~~ distribution was subsequently assessed through~~by~~ flow cytometry analysis (Figure. 4A). Our results showed that in PC3 cells, when SPOCK1 was depleted, cell population~~percentage~~ in G0/G1 phase was significantly increased from 40% to nearly 70%, whereas~~while~~ cell ~~percentage~~ population in S phase and G2/M phase was decreased accordingly ~~decreased~~. On the contrary, when SPOCK1 was up-regulated in RWPE-1 cells, the cell percentage in G0/G1 phase was decreased, which was associated with increased cell proportion in S and G2/M phases (Figure. 4B). The~~Consistently, the~~ critical regulators for cell cycle progression, such as Cdc25C, cyclin~~Cyclin~~ B1, and cyclin~~Cyclin~~ D1, were all consistently altered in response to SPOCK1 expression (Figure. 4C); this outcome confirmed~~), confirming~~ the notion of SPOCK1-mediated regulation of cell cycle progression. ~~More interestingly~~Furthermore, we assessed the role of SPOCK1 in cell apoptosis in PC3 cells ~~with or without SPOCK1 knockdown~~. We found that when SPOCK1 was depleted, cell apoptosis was significantly promoted as compared with control PC3 cells (Figure. 4D). ~~Similarly,~~ SPOCK1-depleted PC3 cells exhibited more ~~severe~~ nuclear fragmentation and chromatin condensation, which represented the apoptotic process. Apoptotic cell quantification~~Quantification of apoptotic cells~~ revealed that shSPOCK1-treated PC3 cells were remarkably apoptotic with the cell apoptosis rate as high as 8% (Figure. 4E). These data suggest that SPOCK1 expression ~~modulation of SPOCK1~~

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~~interrupted~~regulates cell cycle progression and loss of SPOCK1 promotes prostate cancer apoptosis~~affected cell survival~~.

SPOCK1 depletion inhibited~~affected~~ tumor growth in prostate tumor~~a~~ mouse model~~.~~

To test the effects of SPOCK1 modulation on tumor growth *in vivo*, we established a human prostate tumor xenograft~~–~~ mouse model of~~ human prostate cancer~~. Tumors were all dissected on the fourth~~4~~th week. Tumor ~~It was shown that tumor~~ size was shown to be visually smaller in PC3-depleted mouse group~~ of mice~~. On the contrary, tumor sizes in SPOCK1-overexpressed group were markedly greater than those in~~the~~ vector-injected control mouse group~~mice~~ (Figure. 5A). Periodic monitoring~~monitor~~ of tumor volume also showed that SPOCK1 depletion ~~of SPOCK1~~ significantly slowed down tumor growth since the second week. By the fourth~~4~~th week, tumor volume in shSPOCK1 group was only approximately 30% of the shNC group (Figure. 5B). The reverse effects were~~as~~ observed in SPOCK1-overexpressed RWPE-1-derived xenograft tumors~~ cells~~ (Figure. 5C). The effects of tumor growth promotion by SPOCK1 overexpression was also confirmed by the IHC staining of PCNA, which is a ~~marker of~~ cell proliferation marker. ~~With the use of the mouse tumor samples, W~~we performed histological and IHC analysis in the xenograft tumors. IHC staining of PCNA revealed that this proliferation marker was markedly absent in SPOCK1-depleted tumor tissues, whereas this marker~~it~~ was strongly stained in SPOCK1-overexpressed tumor tissues. Expression of cleaved-caspase-3, which is a ~~marker of~~ cell apoptosis marker, went the opposite way as compared with PCNA (Figure. 5D~~). These results supported~~~~),~~ ~~reinforcing~~ the findings that proliferation was inhibited and apoptosis was promoted by SPOCK1 depletion. Furthermore, western blot ~~immunoblot~~ analysis of other apoptosis-related proteins, which include~~including~~ Bad, Bcl-xL, and Bcl-2, showed that the pro-apoptotic factor Bad was ~~negatively~~ downregulated after~~by~~ SPOCK1 overexpression, whereas~~while~~ anti-apoptotic factors, Bcl-xL and Bcl-2, were ~~positively~~ upregulated by SPOCK1 overexpression~~in both PC3 cells and RWPE-1 cells~~. ~~Phosphorylation of~~ AKT (p-AKT) and PI3K (p-PI3K) phosphorylation represents two critical pathways that

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phosphorylate Bad and lead to its inactivation ~~[17, 18].~~ [17, 18] We also found that p-PI3K and p-AKT was positively regulated by SPOCK1 ~~as well~~ (Figure 5E). All these data strongly suggest~~ed~~ that SPOCK1 promot~~sed~~ tumor growth and inhibit~~sed~~ cell apoptosis *in vivo*.

SPOCK1 promoted metastasis in prostate cancer~~.~~

Afterward~~Next~~, we assessed whether SPOCK1 controlled the metastasis process in prostate cancer. Transwell assay analysis showed that SPOCK1-depleted PC3 cells exhibited remarkably ~~decreased~~ attenuated migration and invasion abilities. On the contrary, SPOCK1 overexpression ~~of SPOCK1~~ in RWPE-1 cells caused highly active migration and invasion (Figure. 6A). In fact, in the migration assay, nearly half of the PC3 cells were inhibited from migration when SPOCK1 was depleted; whereas a 60%~~an~~ increase ~~of 60%~~ in migration ability was observed for RWPE-1 cells. Likewise, nearly 70% of PC3 cells were inhibited from invasion~~invading~~ after SPOCK1 knockdown ~~of SPOCK1~~; whereas a 180%~~an~~ increase ~~of 180%~~ in invasion ability was achieved through~~by overexpressing~~ SPOCK1 overexpression in RWPE-1 cells (Figure. 6B). Furthermore, we ~~injected~~ inoculated an equal amount of PC3 cells with (shSPOCK1 group) or without shSPOCK1 (shNC group) into mice through caudal vein (n = ~~=~~10 ~~for each~~per group). ~~Our results~~It showed that in the shNC group, five~~5~~ mice exhibited lung nodules (50% metastasis rate), whereas~~while~~ none of the mice in shSPOCK1 group exhibited nodules in the lung (Figure. ~~–~~6C). These findings led~~),~~ ~~leading us~~ to our conclusion~~conclude~~ that SPOCK1 promoted metastasis both *in vitro* and *in vivo*. In addition, we also detected expression of MMPs, which are critical for cancer cell metastasis. ~~of cancer cells. Consistently,~~ MMP3 and MMP9 were both down-regulated consistently by SPOCK1 knockdown, and were both up-regulated consistently by SPOCK1 overexpression (Figure. 6D). All these conclusive data suggests that SPOCK1 ~~could~~ promotes prostate cancer cell metastasis.

Discussion

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Prostate cancer is the most commonly diagnosed cancer among male patients in many countries and accounts for approximately one in six of ~~all~~ male cancer mortality in ~~the year~~ 2009 (i.e., 124 deaths per 100,000 males). Prostate~~Incidence of prostate~~ cancer incidence~~is~~ steadily increases~~increasing~~ and is reported in almost all countries ~~[19]~~, [19] mainly because of prostate cancer ~~largely due to the~~ metastasis ~~of prostate cancer [3]~~. [3] A number of studies have documented the association between extracellular matrix gene *SPOCK1* and cancer cell metastasis ~~[10, 13, 14]~~; [10, 13, 14] these studies suggest~~,~~ ~~suggesting~~ the extensive role of SPOCK1 in human tumorigenesis.

The ~~present~~ current study investigated the critical roles of SPOCK1 in prostate tumor growth and metastasis ~~in prostate cancer~~. ~~Expression of~~ SPOCK1 expression was initially found to be fairly high in prostate cancer tissues as compared with non-cancerous tissues. In particular, SPOCK1~~Particularly,~~ expression ~~of SPOCK1~~ was higher in metastatic tissues relative to non-metastatic ones. A previous study with microsrray analysis has reported that SPOCK1 was~~SPOCK1was~~ up-regulated or remained unchanged in prostate cancer ~~[15]~~. [15]Another report stated that the ~~up-regulation of~~ SPOCK1 upregulation paralleled that of EPB41L4B, which is a cortical cytoskeleton protein that underlies~~underlie~~ the cell membrane ~~[16]~~. [16] These data would implicate that SPOCK1 might be involved in cell–~~–~~cell adhesion. Furthermore, our results showed that SPOCK1 knockdown ~~of SPOCK1~~ in PC3 cells significantly ~~slowed down~~inhibited cell proliferation, colony formation *in vitro*, and tumor growth *in vivo*; whereas SPOCK1 overexpression ~~of SPOCK1~~ in RWPE-1 cells ~~accelerated~~ promoted cell proliferation and colony formation as well as ~~promoted~~ tumor growth in the mouse model. The~~Knockdown of~~ SPOCK1 knockdown in PC3 cells ~~even~~ arrested cell cycle progression in G0/G1 phase and induced significant cell apoptosis. Cyclin B1, cyclin~~Cyclin~~ D1, and Cdc25C are critical cell cycle regulators that~~which~~ promote checkpoint transitions during cell cycle progression ~~[20-22]~~. [20-22] ~~It was observed that~~ Cyclin B1, cyclin~~Cyclin~~ D1, and Cdc25C were observed to be all positively regulated by SPOCK1 in both PC3 cells and RWPE-1 cells. These~~is~~ results~~finding reinforced~~ support the notion that SPOCK1 regulated cell cycle progression in prostate cancer.

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Another interesting finding was that SPOCK1 promoted metastasis in prostate cancer. SPOCK1 is a glycoprotein that belongs to the extracellular matrix and implicated involving in cell-~~-~~cell adhesion. Metastasis requires stepwise processes that include~~including~~ specialized parameters of cell motility, such as adhesion, chemotaxis, and invasion ~~[23]~~. [23] By~~While~~ employing two distinct approaches, i.e., ~~(~~shRNA for knockdown and expression plasmid for upregulation,~~up-regulation)~~ to modulate SPOCK1 expression ~~of SPOCK1~~, our study showed that SPOCK1 promoted cell migration and invasion *in vitro*. Moreover, SPOCK1 depletion ~~of SPOCK1~~ in PC3 cells directly caused no lung nodules in the experimental mice. These results are conclusive that SPOCK1 ~~mediates~~ promotes prostate cancer cell metastasis. In fact, as an extracellular matrix protein, SPOCK1 has been implicated in the metastasis of gallbladder cancer and hepatocellular carcinoma ~~[10, 14]~~. [10, 14] The finding that~~of~~ SPOCK1 ~~as a promoter for~~promotes prostate cancer metastasis ~~would~~ suggests the extensive role of SPOCK1 in the malignant progression in human cancers.

However, the detailed mechanisms that underlie~~underlying~~ SPOCK1-mediated prostate cancer metastasis remain to be elucidated. One hypothesis would be that SPOCK1 regulated EMT process during cancer metastasis. The following four~~Four~~ steps are required for EMT:~~, including~~ 1) loss of tight junctions, adhesive junctions, and desmosomes; 2) cytoskeletal changes; 3) transcriptional shift; and 4) increased migration and motility. ~~Interruption of~~ EMT interruption is widely recognized as an essential step for cancer distal metastasis ~~[24]~~. [24] MMP3 and MMP9, for instance, are two mesenchymal markers that~~which~~ promoted EMT and, hence, distal metastasis ~~[25, 26]~~. [25, 26] We observed that SPOCK1 positively regulated MMP3 and MMP9 in both PC3 cells and RWPE-1 cells, respectively.~~.~~ This finding may~~might~~ be ~~an~~ evidence~~one clue~~ that indicated the EMT regulation ~~of EMT~~ by SPOCK1 in prostate cancer. Other supportive evidence included that SPOCK1 regulated the EMT process in lung cancer [12] and SPOCK1-mediated EMT signaling conferred~~confered~~ acquired resistance to lapatinib in HER2-positive gastric cancer ~~[13]~~. [13] Therefore, SPOCK1-regulated EMT ~~signaling~~ process might explain why SPOCK1 promotes distal metastasis in prostate cancer. However, our hypothesis is still speculative and

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requiresneeds extensive functional studies for final validation.

SPOCK1The identification, of SPOCK1 as a key mediator of prostate cancer progression, is of great biological significance. Besides, SPOCK1 is also an AR dependent gene and AR signaling continues to be active in almost all stages of prostate cancer. The targeting of SPOCK1 may supplement the therapy with AR antagonist in Prostate Cancer. SPOCK1 was initially isolated from the testes. Our findings may suggest that critical roles of SPOCK1 in the development of genital system disease developmentdiseases. More importantly, SPOCK1 has always been implicated in human cancer progression. Our data may confirm that SPOCK1 exerts extensive oncogenic activities in human tumorigenesis.

In summaryall, we identified that SPOCK1 played critical roles in tumor growth and metastasis in prostate cancer. AlthoughThough the detailed mechanisms remain to be elucidated, the critical role of SPOCK1 in prostate cancer may provide evidence for developmentdevelopments of novel therapeutics against SPOCK1 for the treatment and early detection of prostate cancer.

Figure legends

Figure 1. SPOCK1 is aberrantly overexpressed in prostate cancer tissues. (A) qRT-PCR analysis of SPOCK1 mRNA levels in 20 cases of human prostate cancer. Levels of SPOCK1 mRNA in tumor and the adjacent non-tumor tissues were detected and compared. (B) IHC analysis of the protein expression of SPOCK1 in 50 cases of prostate cancer patients. Representative images showing with the high staining signals of SPOCK1 in tumor tissues were shown. (C) After the scoring of IHC staining, all the 50 tumor tissuecases and 50 non-canceroustumor casetissues were classified into each groupscore. Staining scores of SPOCK1 in the tumor tissues were significantly higher than the non-cancerous tumor tissues. (D) The 50 cases were divided by metastasis (n=24) or not (n=26). It was further shown by IHC analysis that the average staining score of SPOCK1 in metastatic tissues was significantly higher than the non-metastatic tissues. *, P < 0.05;, ***, P < 0.001 as indicated.

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Figure 2. ~~Successful modulation of~~ SPOCK1 stable knockdown or expression in prostate cancer cells. (A) ~~Immunoblot~~ Western blot analysis of the protein levels of SPOCK1 in 5 prostate cancer cell lines. The ~~protein level~~expression of SPOCK1 was highest in PC3 cells, while ~~it was~~ the lowest~~least~~ ~~express~~expression~~ed~~ was in RWPE-1 cells. (B, C) transfection of specific shRNA ~~against~~ targeting SPOCK1 (shSPOCK1) decreased the mRNA level of SPOCK1 in PC3 cells (B), while transfection of its expression plasmid increased its mRNA level in RWPE-1 cells (C). (D) Western blot~~immunoblot~~ analysis further confirmed that ~~the protein level of~~ SPOCk1 was decreased by transfection of shSPOCK1 and increased by transfection of SPOCK1 plasmids in protein levels. **, $p < 0.01$. ***, $p < 0.0001$.

Figure 3. ~~Modulation of~~ SPOCK1 ~~expression affected~~promotes cell proliferation *in vitro*. (A, B) Effects of SPOCK1 knockdown in PC3 cells (A) and overexpression in RWPE-1 cells (B) on cell viability with~~on a~~6 consecutive ~~of 6 days~~day observation. Colony formation ability was assessed after ~~modulation~~ knockdown or overexpression of SPOCK1 in prostate cancer cells. Colony was stained and visualized with crystal violet (C). Quantification of the colonies showed that knockdown of SPOCK1 in PC3 cells significantly decreased, whereas ~~up-regulation~~overexpression of SPOCK1 in RWPE-1 cells increased the number of colonies (D). **, $p < 0.01$.

Figure 4. ~~Modulation of~~ SPOCK1 ~~interrupted~~ knockdown causes cell cycle ~~progression~~ arrest and cell apoptosis ~~process~~. (A, B) cell cycle distribution assessment showed that knockdown of SPOCK1 in PC3 cells induced cell ~~accumulation~~ cycle arrest in G0/G1 phase. Overexpression of SPOCK1 in RWPE-1 cells decreased the cell ~~proportion~~ population in G0/G1 phase, but increased cell ~~percentage~~ population in S phase and G2/M phase. (C) ~~Immunoblot~~Western blot analysis of the critical cell cycle regulators. In ~~SPOCK1-depleted~~ PC3 cells with SPOCK1 knockdown, Cdc25C, Cyclin B1 and Cyclin D1 were consistently decreased. However, in ~~SPOCK1-overexpressed~~ RWPE-1 cells with SPOCK1 overexpression, expression of Cdc25C, Cyclin B1 and Cyclin D1 were increased. (D) Annexin-PI

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analysis of cell apoptosis in PC3 ~~with or without SPOCK1 depletion~~. When SPOCK1 was knocked down ~~depleted~~, cell apoptosis rate was increased to 10%, while it was less than 5% in the control PC3 cells. (E) Detection of morphological changes in apoptosis with Hoechst 33342 staining. SPOCK1-depleted PC3 cells exhibited more obvious~~severe~~ nuclear fragmentation and chromatin condensation. The apoptosis rate was significantly higher compared with~~than~~ the control cells (8% vs. 1%). **, $p < 0.01$. ***, $p < 0.0001$.

Figure 5. SPOCK1 ~~affected~~ promotes tumor growth in xenograft prostate ~~a mouse~~tumor mouse model. (A) Tumor dissection showed that knockdown of SPOCK1 ~~caused tumor size smaller~~decreases tumor voloumn, while overexpression of SPOCK1 ~~enlarged tumor sizes~~increases. (B, C) periodic monitoring of tumor volume in PC3 cell derived tumors~~s~~ or~~and~~ RWPE-1 cell derived ones~~s~~ in ~~a consecutive of 4~~4 consecutive weeks. (D) Histology and immunohistochemistry analysis of the tumor tissue sections from the mouse model. Proliferating cell nuclear antigen (PCNA)~~, a proliferation marker,~~ and cleaved-caspase-3 were detected for indicating cell proliferation and apoptosis, respectively. (E) ~~Immunoblot~~ Western Blot analysis of ~~expression of~~ SPOCK1 expression and a series of apoptosis-related ~~proteins~~markers. ~~It was observed that~~ SPOCK1 ~~positively~~ upregulated anti-apoptotic factors Bcl-2 and Bcl-xL as well as phophorylation kinases of Bad such as p-PI3K and p-AKT. The pro-apoptotic factor Bad was ~~negatively~~ downregulated by SPOCK1 knockdown in both PC3 cells and RWPE-1 cells.

Figure 6. SPOCK1 promoted metastasis in prostate cancer. (A) Transwell assay showed that ~~SPOCK1 depleted~~ PC3 cells with SPOCK1 knockdown exhibited remarkably decreased migration and invasion abilities; whereas overexpression of SPOCK1 in RWPE-1 cells ~~caused~~ enhanced~~highly active~~ cell migration and invasion. (B) Quantification of the transmigrated cells in the Transwell assay~~.~~ ~~*~~, ~~*~~*, $p < 0.01$. (C) ~~Injection~~ Inoculation of PC3 cells into two groups of mice (n=10 ~~for each~~per group) ~~through~~ via caudal vein. PC3 cells were pre-transfected with shSPOCK1 or not. It was

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observed that no mice in SPOCK1-depleted group exhibited lung metastatic lesions~~nodules~~. (D) ~~immunoblot~~ Western blot analysis of matrix metallproteases (MMPs). The MMP3 and MMP9 were either~~both~~ ~~positively~~ upregulated or ~~by SPOCK1 in~~downregulated in PC3 cells with SPOCK1 overexpression or~~and~~ RWPE-1 cells with its knockdown.