SPOCK1 promotes tumor growth and metastasis in human prostate cancer

Abstract
Prostate cancer is the most diagnosed non-cutaneous cancer and ranks as the leading cause of cancer-related deaths in American males. 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 cancer. Molecul...
of tumor growth and metastasis in prostate cancer.

**Keywords**: SPOCK1; tumor progression; tumor metastasis; prostate cancer.

**Introduction**

Prostate cancer is the most diagnosed non-cutaneous cancer and ranks as the second leading cause of cancer-related deaths in American males [1]. Based on a recent statistics, there were 238,590 newly 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 the most serious health problem among male patients [2]. Metastasis is the primary factor for prostate cancer mortality deaths of this malignancy [3]. 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 is nearly 100% for localized patients [4]. The even worse finding is that, the overall survival has not changed in the last 20 years among patients who suffer from metastatic prostate cancer. However, though an approximately 40% decrease in the mortality of this malignancy has been achieved over the last two decades. Hence, the meanshow to prevent prostate cancer progression and to perform necessary interventions make early interference before this cancer metastasizes to other organs remains a major clinical challenge.

SPARC/osteonectin, cwcv, and kazal-like domain 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 shares a similar structure and homologous domains, are implicated in cell proliferation, cell-cell adhesion, and migration [5]. SPOCK1 has been observed to play crucial roles in cell cycle regulation, cell apoptosis, DNA repair, and metastasis [6]. Expression of SPOCK1 was fairly high in the brain [2]. This proteoglycan is also present...
SPOCK1 is overexpressed in prostate cancer tissues.

Initially, we performed qRT-PCR analysis of the SPOCK1 mRNA levels in 20 consecutive cases of prostate cancer cases. Our data showed that the relative mean mRNA level of SPOCK1 in the cancerous tissues was approximately two-fold 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 proteoglycan was rarely detected in non-tumor tissues (Figure 1B). Further analysis showed that 32 of the 50
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 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 level in RWPE-1 cells (Figure 2A). This result 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 upregulated 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 transfection of SPOCK1 plasmid 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 transfection of its expression plasmid (Figure 2D). These data confirmed the successful construction of prostate cancer cell lines that were stably depleted with either stable knockdown of SPOCK1 (PC3 cells) or overexpression of SPOCK1 (RWPE-1 cells).

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 decreased
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 also performed colony formation assay (Figure 3C). The SPOCK1 knockdown was shown to significantly decrease 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 was subsequently assessed through flow cytometry analysis (Figure 4A). Our results showed that in PC3 cells, when SPOCK1 was depleted, cell population in G0/G1 phase was significantly increased from 40% to nearly 70%, whereas cell population in S phase and G2/M phase was decreased accordingly. On the contrary, when SPOCK1 was up-regulated in RWPE-1 cells, cell population in G0/G1 phase was decreased, which was associated with increased cell proportion in S and G2/M phases (Figure 4B). The critical regulators for cell cycle progression, such as Cdc25C, cyclin B1, and cyclin D1, were all consistently altered in response to SPOCK1 expression (Figure 4C); this outcome confirmed the notion of SPOCK1-mediated regulation of cell cycle progression. 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 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
interrupted regulates cell cycle progression and loss of SPOCK1 promotes prostate cancer apoptosis-affected cell survival.

SPOCK1 depletion inhibited tumor growth in prostate tumor 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 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 vector-injected control mouse group (Figure 5A). Periodic monitoring of tumor volume also showed that SPOCK1 depletion of SPOCK1 significantly slowed down tumor growth since the second week. By the fourth week, tumor volume in shSPOCK1 group was only approximately 30% of the shNC group (Figure 5B). The reverse effects were 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, we performed histological and IHC analysis. IHC staining of PCNA revealed that this proliferation marker was markedly absent in SPOCK1-depleted tumor tissues, whereas this marker 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 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 SPOCK1 overexpression, whereas 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
phosphorylate Bad and lead to its inactivation \cite{17, 18}. We also found that p-PI3K and p-AKT was positively regulated by SPOCK1 as well (Figure 5E). All these data strongly suggested that SPOCK1 promoted tumor growth and inhibited cell apoptosis \textit{in vivo}.

\textbf{SPOCK1 promoted metastasis in prostate cancer.}

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

\textbf{Discussion}
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 cancer incidence is steadily increasing and is reported in almost all countries\(^1\), \(^{19}\) mainly because of prostate cancer largely due to the metastasis of prostate cancer\(^2\). \(^3\) A number of studies have documented the association between extracellular matrix gene \textit{SPOCK1} and cancer cell metastasis\(^{10, 13, 14}\); these studies suggest, suggesting the extensive role of SPOCK1 in human tumorigenesis.

The present study investigated the critical roles of SPOCK1 in prostate tumor growth and metastasis in prostate cancer. Expression of SPOCK1 was initially found to be fairly high in prostate cancer tissues as compared with non-cancerous tissues. In particular, SPOCK1 expression was higher in metastatic tissues relative to non-metastatic ones. A previous study with microarray analysis has reported that SPOCK1 was up-regulated or remained unchanged in prostate cancer\(^{15}\). Another report stated that the up-regulation of SPOCK1 paralleled that of EPB41L4B, which is a cortical cytoskeleton protein that underlies the cell membrane\(^{16}\). These data would implicate that SPOCK1 might be involved in cell-cell adhesion. Furthermore, our results showed that SPOCK1 knockdown in PC3 cells significantly inhibited cell proliferation, colony formation in vitro, and tumor growth in vivo; whereas SPOCK1 overexpression in RWPE-1 cells accelerated cell proliferation and colony formation as well as promoted tumor growth in the mouse model. The knockdown of SPOCK1 in PC3 cells even arrested cell cycle progression in G0/G1 phase and induced significant cell apoptosis. Cyclin B1, cyclin D1, and Cdc25C are critical cell cycle regulators that promote checkpoint transitions during cell cycle progression\(^{20-22}\). It was observed that Cyclin B1, cyclin D1, and Cdc25C were all positively regulated by SPOCK1 in both PC3 cells and RWPE-1 cells. These results reinforce the notion that SPOCK1 regulated cell cycle progression in prostate cancer.
Another interesting finding was that SPOCK1 promoted metastasis in prostate cancer. SPOCK1 is a glycoprotein that belongs to the extracellular matrix and implicated in cell–cell adhesion. Metastasis requires stepwise processes that include specialized parameters of cell motility, such as adhesion, chemotaxis, and invasion [23]. By employing two distinct approaches, i.e., shRNA for knockdown and expression plasmid for upregulation to modulate SPOCK1 expression, our study showed that SPOCK1 promoted cell migration and invasion in vitro. Moreover, SPOCK1 depletion directly caused no lung nodules in the experimental mice. These results are conclusive that SPOCK1 mediates 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]. The finding that SPOCK1 as a promoter for prostate cancer metastasis would suggest the extensive role of SPOCK1 in the malignant progression in human cancers.

However, the detailed mechanisms 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 steps are required for EMT: 1) loss of tight junctions, adhesive junctions, and desmosomes; 2) cytoskeletal changes; 3) transcriptional shift; and 4) increased migration and motility. Interruption of EMT is widely recognized as an essential step for cancer distal metastasis [24]. MMP3 and MMP9, for instance, are two mesenchymal markers that promoted EMT and, hence, distal metastasis. We observed that SPOCK1 positively regulated MMP3 and MMP9 in both PC3 cells and RWPE-1 cells. This finding may indicate 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 acquired resistance to lapatinib in HER2-positive gastric cancer [13]. Therefore, SPOCK1-regulated EMT signaling might explain why SPOCK1 promotes distal metastasis in prostate cancer. However, our hypothesis is still speculative and
The 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 the critical roles of SPOCK1 in the development of genital system disease. 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 summary, we identified that SPOCK1 played critical roles in tumor growth and metastasis in prostate cancer. Although the detailed mechanisms remain to be elucidated, the critical role of SPOCK1 in prostate cancer may provide evidence for development 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 the high staining signals of SPOCK1 in tumor tissues were shown. (C) After the scoring of IHC staining, all the 50 tumor tissues and 50 non-cancerous tumor cases were classified into each group. Staining scores of SPOCK1 in the tumor tissues were significantly higher than the non-cancerous 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.
**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 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 affects cell proliferation *in vitro*. (A, B) Effects of SPOCK1 knockdown in PC3 cells (A) and overexpression in RWPE-1 cells (B) on cell viability within 6 consecutive days of 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 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-P1
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 the control cells (8% vs. 1%). **, p < 0.01. ***, p < 0.0001.

Figure 5. SPOCK1 affects tumor growth in xenograft prostate tumor mouse model. (A) Tumor dissection showed that knockdown of SPOCK1 caused tumor size smaller decreases tumor volume, while overexpression of SPOCK1 enlarged tumor sizes increases. (B, C) Periodic monitoring of tumor volume in PC3 cell-derived tumors and RWPE-1 cell-derived ones in consecutive 4 week 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 and a series of apoptosis-related proteins. It was observed that SPOCK1 positively upregulated anti-apoptotic factors Bcl-2 and Bcl-xL as well as phosphorylation kinases of Bad such as p-PI3K and p-AKT. The pro-apoptotic factor Bad was negatively down regulated by SPOCK1 knockdown in both PC3 cells and RWPE-1 cells.

Figure 6. SPOCK1 promotes 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 per group) through via caudal vein. PC3 cells were pre-transfected with shSPOCK1 or not. It was
observed that no mice in SPOCK1-depleted group exhibited lung metastatic lesions nodules. (D) immunoblot-Western blot analysis of matrix metalloproteases (MMPs). The MMP3 and MMP9 were either both positively upregulated or both downregulated in PC3 cells with SPOCK1 overexpression or and RWPE-1 cells with its knockdown.