Exogenous SPARC Suppresses Proliferation and Migration of Prostate Cancer by Interacting With Integrin β1

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BACKGROUND. The matricellular protein secreted protein acidic and rich in cysteine (SPARC) plays an important role on tumor metastasis and progression in several cancers. However, the roles of SPARC in prostate cancer (PCa) remain unclear.
METHODS. To identify SPARC protein in prostate tissue, immunohistochemical analysis of SPARC was conducted using human prostate tissue microarray. To detect SPARC expression in prostate cancer (LNCaP, DU145, and PC-3) and stromal cells, RT-PCR, western blot analysis, and ELISA was conducted. To reveal the function of exogenous SPARC in PCa cells, AKT phosphorylation was confirmed by western blot analysis after coculture with stromal cells. Proliferation and migration of PCa cells were examined by addition of SPARC. The interaction between SPARC and integrin β1 was confirmed by western blot analysis after immunoprecipitation.
RESULTS. SPARC protein was expressed well in normal tissue compared with PCa tissue. ELISA showed high secreted SPARC protein in normal prostate-derived stromal cell (PrSC) compared with PCa-derived stromal cell (PCaSC) and PCa. PCa cells cocultured with PrSC showed reduced AKT phosphorylation more than with PCaSC. PCa cells cocultured with PrSC whose SPARC was knocked-down restored AKT phosphorylation. Moreover, PCa cells treated with SPARC led to reduced AKT phosphorylation. Immunoprecipitation with SPARC revealed interaction of SPARC and integrin β1 in PCa cells. Inhibited proliferation and migration of PCa cells by SPARC was restored by integrin β1 neutralizing antibody.
CONCLUSIONS. Reduced SPARC secretion from stromal cells might affect PCa progression mediating through limiting AKT phosphorylation after interaction with integrin β1.
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KEY WORDS: **prostate cancer; SPARC; stromal cells; integrin β1**

INTRODUCTION

Prostate cancer (PCa) is a common malignancy in men worldwide and the second leading cause of cancer related death among the male population of the United States [1]. It has been determined that advanced PCa cells usually metastasizes to bone [2] and lymph nodes [3]. Tumor metastasis and growth occur in the context of extracellular matrix (ECM) and require an interaction of malignant cells with various

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microenvironments [4,5]. As matricellular proteins in the ECM mediate the interactions between cell–cell and cell–extracellular environment, understanding the interaction of cancer cells with their microenvironment has emerged as an essential step.

Secreted protein acidic and rich in cysteine (SPARC)/osteonectin/BM-40 is a matricellular glycoprotein that plays instrumental roles during cell proliferation, immune response, migration, and cell differentiation modulating reversible interactions between cells and ECM [6]. Several proteinases, including matrix metalloproteinases (MMPs), release bioactive fragments from SPARC that also affect angiogenesis and cell behavior [7]. In tumorigenesis, SPARC interacts with an ECM and is associated with tumor cell growth, differentiation, metastasis and invasion [8–10]. A secreted glycoprotein, SPARC binds to several integral components of the ECM. Although high-affinity SPARC receptor has not been identified [11], numerous studies suggest that SPARC regulates integrin signaling and the ability of integrins to interact with structural components of the ECM [12,13]. Recent studies have shown that SPARC-knockout mice grow cancers faster than mice expressing SPARC [14,15] and show accelerated wound healing [16,17]. SPARC acts not as a tumor suppressor but also as a promoter of invasiveness through integrin axis in melanoma, and was studied as a molecular marker, which represent a highly aggressive phenotype [18,19]. SPARC takes different contradictory actions depending on cell-type and context showing contradictory effects on tumor progression.

The role of SPARC in PCa is also not fully understood yet. SPARC has been reported to be predominantly tumorigenic in PCa cells [20,21]. In contrast, recent studies with homozygous SPARC KO/TRAMP model has been reported that SPARC was limiting for primary prostate tumorigenesis and progression [22]. Kapinas et al. [23] identified bone matrix-associated SPARC limited proliferation of PCa cells and increased their sensitivity to ionizing radiation. Since tumors are heterogeneous population, effects of SPARC on PCa cells are the results of a crosstalk between PCa cells and microenvironments.

In that respect, we aim to provide insight into how SPARC might affect PCa cells. We investigated how differentially expressed SPARC between normal prostate derived stromal cell (PrSC) and PCa-derived stromal cell (PCaSC) affect the progression of PCa cells. We also confirmed an interaction between SPARC and integrin β1, which may define the signaling mechanism for migration and proliferation of PCa cells.

MATERIALS AND METHODS

Immunohistochemistry of SPARC

For tissue microarrays (TMAs), we purchased from Isu Abxis Co. Ltd. (South Korea) and Provitro (Berlin, Germany). The procedure for immunohistochemical staining (IHC) was performed using a Dako ChemMate ENVISION Kit/HRP(DAB)-universal kit (K5007) according to the manufacturer's protocol (Dako, Carpinteria, CA). After blocking endogenous peroxidase activity using 3% hydrogen peroxide for 10 min, the sections were incubated with rabbit monoclonal antibodies against SPARC (R&D system) at a dilution of 5 μg/200 μl at room temperature for 60 min. After the primary antibodies reaction, sections were washed with PBS slowly and followed by peroxidase labeled secondary antibody (a mixture of rabbit and mouse antibodies) combined with dextranpolymers. Brownish staining for target proteins on tissue slides was developed using DAB within 5 min, and counterstained with hematoxylin. Images were captured on microscope ×40 and ×100.

Cell Lines, Reagents, and Transfections

DU145, LNCaP (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) and 5% fetal bovine serum (FBS; Sigma–Aldrich, St. Louis, MO) and PC-3 (American Type Culture Collection) were cultured in RPMI1640-5% FBS. Normal prostate-derived stromal cells, PrSC, commercially available (Cambrex, East Rutherford, NJ) were cultured using SCGM Bullet Kit (Cambrex). Human PCaSC were isolated from PCa tissue as described previously [24] and cultured in RPMI1640-10% FBS. Cell cultures were incubated at 37°C with 5% CO_2. For coculture, 0.4 μm pore size transwell was used. PCa cells (LNCaP, DU145, and PC-3) were placed on the bottom of the lower chamber while PrSC and PCaSC were placed on the membrane of the upper chamber. The transwell prevents direct cell–cell interactions but allows the diffusion of soluble factors through the membrane. For treatment with SPARC (Osteonectin, Human platelets; Calbiochem, La Jolla, CA), all cells were serum-starved in medium containing 0.1% FBS for 12 hr, followed by incubation with SPARC or vehicle (media) for 24 hr. Cells were harvested for protein and mRNA detection. For blocking experiments, 10 μg/ml nonspecific IgG, integrin β1-blocking antibody (Millipore, USA) was added to the media during SPARC treatment.

ReverseTranscription-PCR (RT-PCR)

Total RNA was extracted from confluent monolayers of cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was made by reverse-transcription (RT) of 1 μg of each total RNA using cDNA Synthesis Kit (Bio-Rad, USA). Each cDNA sample was amplified with ExTaq (Takara Bio, Japan). The oligonucleotide primer sets used for PCR analysis of cDNA were SPARC, 5′-CGGGACTTCGAGAAGAACTA-3′ (forward) and 5′-AGACCTGTGACCTGGACAAT-3′ (reverse), GAPDH, 5′-CCACCCATGGCAAATTCCATGGCA-3′ (forward) and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (reverse), Integrin α1, 5′-GTGGGCCAACAAAGAACACT-3′ (forward) and 5′-TGGAAGCAGGCCCAAATATAG-3′ (reverse), Integrin α2, 5′-GTTTT GAAAGGCGAGCAAAG-3′ (forward) and 5′-GCTG TTGGCTAAAGGACTCG-3′ (reverse), Integrin β1, 5′-GGCTCTGCTTTGGACAGAAC-3′ (forward) and 5′-ACCACGGAAACAAGGAAGTG-3′ (reverse), Integrin β2, 5-CACAAGCTGGCTGAAAACAA-3′ (forward) and 5′-ATTGCTGCAGAAGGAGTCGT-3′ (reverse). For SPARC, Integrin and GAPDH amplification, the PCR condition was 94°C, 3 min followed by 30 cycles of 94°C, 40 sec; 57°C, 30 sec; 72°C, 30 sec; and 72°C, 3 min final extension. The amplified PCR products were visualized using electrophoresis on a 1.5% agarose gel.

Western Blot Analysis

Cells were lysed in mammalian protein extraction buffer (Pierce, Rockford, IL), and total protein was extracted as described previously [25]. Equal amounts of protein were resolved on a 10% or 12.5% Ready Gel J (Bio-Rad, Hercules, CA), transferred onto a PVDF membrane (Invitrogen), and blocked with 5% non-fat dry milk at room temperature. Membranes were probed overnight with primary antibodies against SPARC (Cell Signaling Technology, Beverly, MA), GAPDH (Novus Biologicals, Littleton, CO), AKT (Cell Signaling), P-AKT (Cell Signaling), SPARC (Cell signaling), and PARP (Cell signaling) followed by HRP-conjugated secondary antibodies. An ECL system was used to detect chemiluminescent signals (SuperSignal West Pico Chemiluminescent Substrate; Pierce).

Enzyme-Linked Immunosorbant Assay (ELISA)

Cells (4×10^5 cells/well, six-well plates) were stimulated with SPARC for 24 hr and the amount of SPARC protein in cell culture media was determined by using commercially available SPARC ELISA kits (Takara Bio). The conventional ELISAs were prepared by coating the bottom of a 96-well plate with capture antibodies. Cell culture media was incubated for one h in antibody coated plate then washed four times with TBST. Equal amounts of substrate solution were added to each well and incubated for 15 min at room temperature. After adding stop solution, absorbance readings were made at 450 nm, using a 96-well plate spectrophotometer.

Cytokine Antibody Array

Secreted SPARC was analyzed with a cytokine antibody array by using a RayBio cytokine antibody array kit (RayBiotech, Inc., Norcross, GA), according to the manufacturer's instructions. Supernatants of cells were incubated with blocked membranes and then incubate with biotinylated detection antibody cocktail and HRP-conjugated streptavidin. Signal intensities were quantified directly with a chemiluminescence imaging system and Image J (NIH, USA) software.

RNA Interference Analysis

The specific SPARC interfering RNAs (siRNA) sequences were designed by Invitrogen. SPARC target siRNA sequence was 5′-UAGAAUUGCAACAGCUUGUCCUUCC-3′. Non-target siRNA (NT siRNA) were purchased from Invitrogen. For SPARC knockdown in PrSC, transfection experiments were performed using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's protocol (Invitrogen). Briefly, cells were transfected with of 5, 10, and 20 nM SPARC siRNA or 20 nM NT siRNA for 24 hr. After 6 hr of transfection, complete medium was added and cells were cultured for another 12 or 24 hr. At the end of the culture period, total RNA were exacted or the cells were transferred to transwell for coculture.

Immunoprecipitation (IP)

After finishing various treatments, cells were lysed in mammalian protein extraction buffer (Pierce) as described under western blot procedure. Immunoprecipitation were performed using Catch and release IP reagents (Abcam, MA) according to the manufacturer's protocol. Briefly, total proteins were quantified according to the method of Bradford, and equal amounts of protein (500 μg) were mixed with 2 μg of rabbit monoclonal antibodies against SPARC (Cell Signaling) and Affinity ligand. After providing a final volume 500 μl, the mixtures were incubated on a rotor mixer at room temperature for 30 min. Unbounded non-specific proteins were washed with the wash buffer and immunocomplexes with Antibody Capture Affinity Ligand (ACAL) were left. From the complex, eluted proteins were subjected to western blot

analysis with integrin β1 antibody (Cell signaling) and for normalization of rabbit monoclonal antibodies against SPARC (Cell Signaling), anti-rabbit HRP-linked antibody (Cell Signaling) was used.

Cell Proliferation Assays

Cell proliferation was determined using a quick cell proliferation assay kit (Abchem) according to the manufacturer's instructions. Cells (3×10^4/well) were plated in 96-well microtiter plate in a final volume of 100 μl/well culture medium in absence or presence of SPARC (μg/ml) integrin β1–blocking antibody (10 μg/ml). After 12, 24, 36, and 72 hr, 10 μl/well WST-1/ECS solution was added and incubated 4 hr in standard culture condition (37°C with 5% CO_2). Plate was shaken thoroughly for 1 min on a shaker. The absorbance of each well was measured at 450 nm with a microtiter plate reader.

Migration Assay

In vitro wound-healing assay was performed as previously described [25]. Cells were incubated in culture medium containing with 1% penicillin/streptomycin and 5% FBS in presence or absence of 0.5 μg/ml SPARC. Wound healing was visualized with photographs by microscope. Cell migration assay using a transwell were performed in migration and invasion chambers according to the manufacturer's instructions (Cell Biolabs, Inc., CA). Before assay, cells were serum-deprived for 24 hr, and cells were placed into upper chamber in serum-free medium. Medium supplemented with 5% FBS was placed in the lower chamber with SPARC (μg/ml) or isotype control and integrin β1-blocking antibody (10 μg/ml), respectively. After incubation, cells that had migrated to the lower surface of the filters were stained and quantified by colorimetric reading at 560 nm.

Statistical Analysis

All data are presented as mean ± SD, and statistical significance was determined by using the Prism 4.0 software. The χ^2 test was utilized to assess the significance between different proportions. Analysis of continuous variables between different groups was assessed by one-way analysis of variance followed by Fisher's protected least significant difference test. Differences were considered to be statistically significant. Relative amount values were expressed as means ± SD from Three replicate experiments. * and ** represent significant difference $P < 0.05$, and $P < 0.01$, respectively. Kruskal-Wallis test was used to determine the statistical significance of differences in IHC staining of tissue microarray.

RESULTS

Distribution of SPARC Between Human Prostate Cancer and Normal Prostate

To investigate the effect of SPARC on human PCa, we first examined the existence of SPARC protein in human prostate. As shown in (Fig. 1A–H), immunohistochemistry using tissue microarrays revealed that SPARC was expressed in 87% normal prostate tissues (28/32) and in 9% of the tumor tissues (4/64; Table I). Normal prostates cells showed strong intensity of SPARC expression compared with prostate caner tissue ($P < 0.001$). Strong staining of SPARC in normal stroma was also observed compared with PCa stroma. We could not observe significant difference in the intensity of SPARC staining among the level of Gleason score statistically.

Differential Expression of SPARC in Prostate Cancer Cell Lines and Prostate Derived Stromal Cell Lines

SPARC is a highly conserved, multifunctional protein that regulates various cell in different ways [10]. To determine the roles of SPARC from prostate stromal cells on PCa cells, we first examined the expression of SPARC mRNA and protein in LNCaP, DU145, and PC-3 cells, PCaSC-5, 8 (PCa-derived stromal cells), and PrSC (normal prostate- derived stromal cells; Fig. 2A,B). Both mRNA and protein level of SPARC (42 kDa) was higher in PCaSC-5, -8, and PrSC) than PCa cells (LNCaP, DU145, and PC-3). Moreover, western blot analysis revealed higher expression of SPARC protein in PrSC than PCaSCs. SPARC has been reported as secreted noncollagenous glycoprotein that closely associated with an ECM [6]. We also examined secreted SPARC in medium from PCa cells, PCaSCs, and PrSC by ELISA (Fig. 2C). Secreted SPARC level from stromal cells was relatively high compared with three kinds of PCa cells. Furthermore, the amount of secreted exogenous SPARC from PrSC was 1.8–2.4 times higher than other PCa-derived stromal cells (PCaSC-5, -6, -7, -8) and 6.8–11.3 times higher than PCa cells (LNCaP, DU145, and PC-3). To evaluate SPARC protein expression levels of cytoplasm, we performed cytokine assay using anti-cytokine specific membrane and compared the level of secreted cytokines from normal prostate stromal cells (PrSC) and PCa-derived stromal cells (PCaSC-5; Fig. 2D). This result was coincident well with ELISA: the lower expression of SPARC in PCaSC-5 was detected compared with normal prostate PrSC. Therefore, we investigated the effect of diminished SPARC expression in PCa stromal cells on PCa cells.

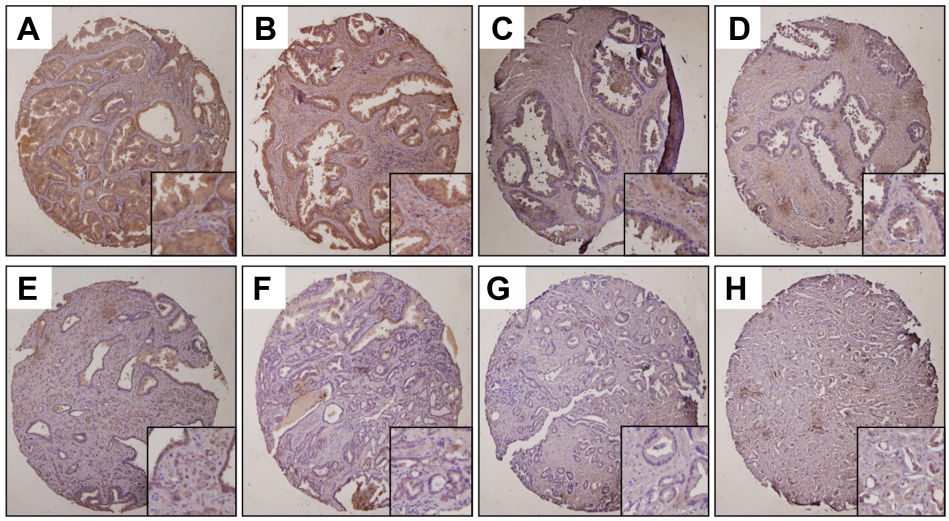

Fig. 1. Immunohistochemical staining of SPARC on prostate tissue. Commercial PCa tissue microarray (TMA) slides (**A**–**H**) were deparaffinized in xylene and rehydrated in graded alcohols and processed as described method. TMAs with 96 specimens and tissue sections were immunostained with SPARC antibody. Representative examples of photomicrographs are showing SPARC expression in the non-neoplastic prostate and PCa. A: non-neoplastic from E. B: non-neoplastic from F. C: non-neoplastic from G. D: non-neoplastic from H. E: Gleason score 7. F: Gleason score 7. G: Gleason score 8. H: Gleason score 9 (original magnification ×40 and ×100).

Exogenous SPARC Reduces AKT Phosphorylation

Since stromal cells, especially normal prostate stromal cells (PrSC) secreted SPARC well compared with PCaSC, we investigated the effect of exogenous SPARC secreted from stromal cells on PCa cells. We first analyzed whether exogenous SPARC induces any change in AKT signal that provides a major therapeutic opportunity in prostate disease [26]. Western blot analysis revealed that exogenous SPARC reduces AKT phosphorylation in a dose-dependent manner in LNCaP, DU145, and PC-3 cells (Fig. 3A). We also performed coculture of PCa cells with stromal cells that secrets higher level of SPARC protein (Fig. 3B). Phosphorylation of AKT was decreased in all PCa cells by coculture with stromal cells in accordance with secreted level of SPARC. Many soluble factors from stromal cells may affect phosphorylation of AKT in PCa cells. In order to confirm reduced phosphorylation in PCa cells is due to secreted SPARC from stromal cells, we performed knockdown of SPARC in PrSC using small interfering RNA. Knockdown of SPARC by siSPARC repressed the expression level of SPARC mRNA and secreted SPARC protein in medium in dose-dependent manner (Fig. 3C). Then coculture of PCa cells with PrSC transfected with siSPARC reduced AKT phosphorylation in all PCa cells (Fig. 3D).

Exogenous SPARC-Integrin β I Interaction Reduces AKT Phosphorylation

Goal et al. has shown that that tumor cells express an abnormal integrin repertoire and surrounded by a markedly aberrant ECM [27]. To reveal the

TABLE I. Immunohistochemistry of SPARAC on ProstateTissue Microarray

Clinicopathological features	SPARC expression (−)	(+)	(++)	Total number	*P*-value
Normal	1	2	19	22	
PIN	2	3	5	10	0.001
Gleason score					
5, 6	7	2	1	10	0.001
7	28	2	0	30	0.001
8, 9, 10	23	1	0	24	0.001
Total number	61	10	25	96	

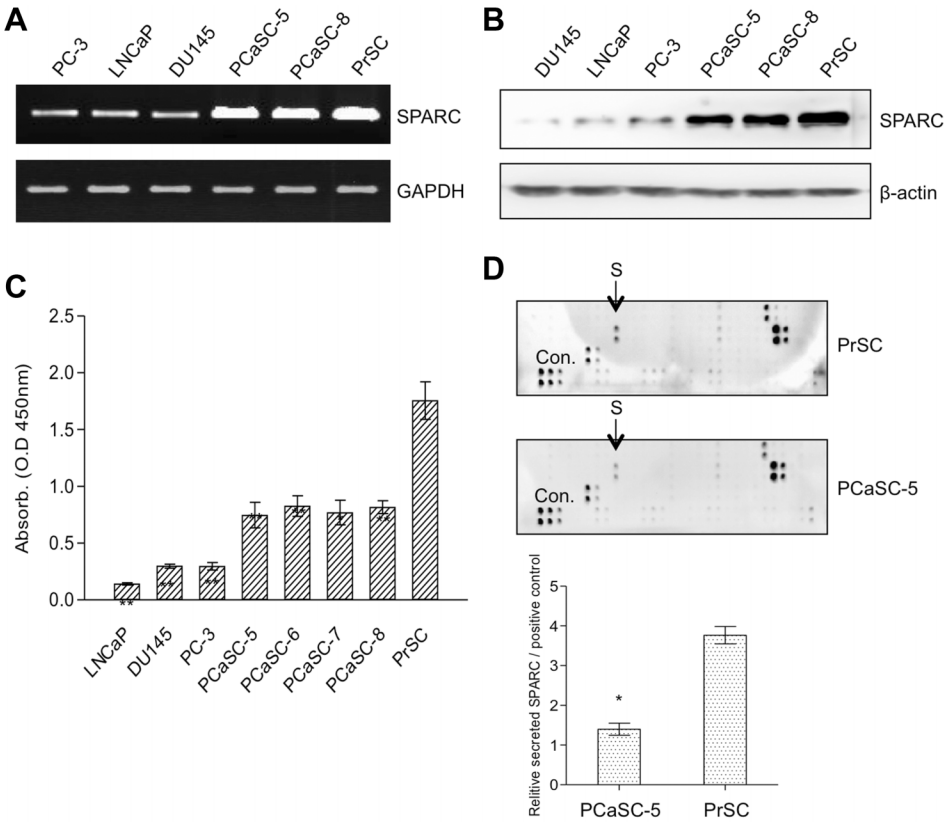

Fig. 2. Expression level of SPARC in PCa cells, PrSC, and PCaSCs. **A**: Expression levels of SPARC mRNA were measured in PCa cell lines (LNCaP, DUI45, and PC-3) and stromal cell lines (PCaSC-5, PCaSC-8, PrSC) by RT-PCR. **B**: Whole cell lysate from each cells were also subjected to Western blot analysis and normalized as a ratio using β-actin. **C**: Each cell (4×10^5) was cultured in serum-free medium for 36 hr and SPARC protein concentrations in the medium were measured by ELISA assay. OD values obtained by ELISA reader at 450 nm are expressed as means ± SD from four replicate experiments. Cells (1×10^6 cells) were cultured with 0.2% FBS for 2 days and the supernates were incubated with commercially available array membrane. **D**: Comparison of Secreted SPARC in PrSC and PCaSC-5. Images were collected using a chemiluminescence imaging system after cytokine array. S represents secreted SPARC (top). The data on the bottom represents relative amount of secreted SPARC.

relationship between exogenous SPARC and integrins, the expression level of integrin mRNA in PCa cells treated with SPARC for 2 days was examined using quantitative RT-PCR (Fig. 4A). Regardless of a dose of SPARC, the expression level of integrin α1, α2, and β2 mRNA was constant. However, the expression level of only integrin β1 mRNA was increased in a dose-dependent manner. To confirm the direct interaction between exogenous SPARC and integrin β1, lysate from LNCaP, DU145, and PC-3 cells that were treated with exogenous SPARC were immunoprecipiated with anti-SPARC antibodies and were subsequently probed for anti-integrin. Although no interaction was detected when immunoprecipitation was probed for anti-integin α1, α2, and β2 (data not shown), exogenous SPARC interacted with integrin β1 (Fig. 4B). To further verify the apparent interaction between SPARC and integrin β1, we pretreated integrin β1-blocking antibody and nonspecific isotype control antibody in LNCaP, DU145, and PC-3 cells, and then treated with SPARC for 2 days. The interaction between integrin β1 and exogenous SPARC was diminished by integrin β1-blocking antibody. Furthermore, after blocking the interaction reduced AKT phosphorylation by exogenous SPARC was recovered (Fig. 4C), suggesting that exogenous SPARC directly binds to integrin β1 and affects AKT signaling.

Exogenous SPARC Decreases Prostate Cancer Cells Proliferation

We analyzed the effect of exogenous SPARC on cell proliferation (Fig. 5A). The proliferation rates of LNCaP, DU145, and PC-3 cells treated with 1 μg/ml SPARC were decreased compared with vehicle. To determine whether inhibition of cell proliferation by

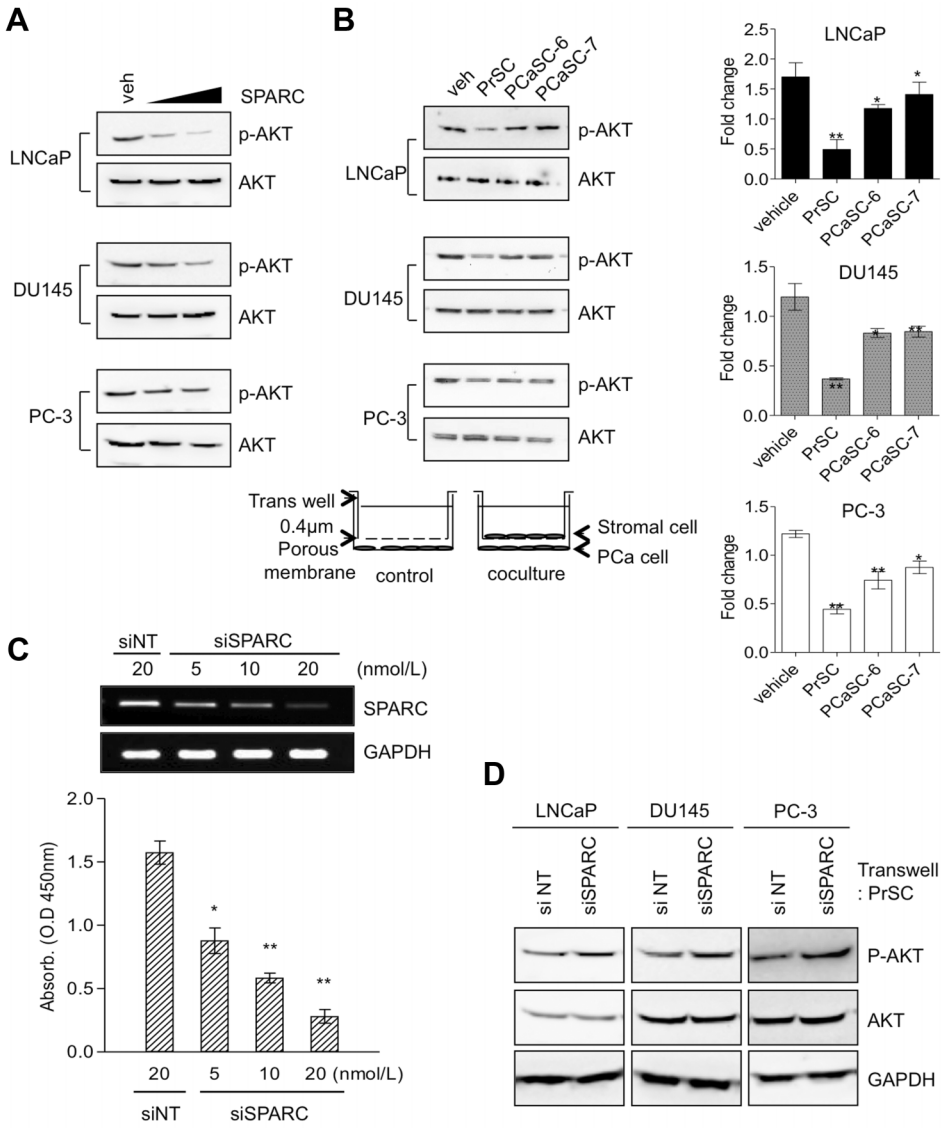

Fig. 3. Inactivation of AKT by exogenous SPARC. **A**: After LNCaP, DUI45, and PC-3 cells (4×10^5) were treated with vehicle (medium) or 0.5, 1.0 μg/ml SPARC for 2 days, whole cell lysate from each sample was also subjected to Western blot analysis. **B**: LNCaP, DUI45, and PC-3 cells were cocultured with vehicle, PrSC, PCaSC-6, and PCaSC-7 for 48 hr in a transwell chamber as illustrated transwell coculture systems. The cell lysates from PCa cells were subjected to western blot analysis with anti-phospho-AKTand anti-AKTantibody (Left panel). Right panel represents relative changes of phospho-AKTexpression. **C**: Knockdown of SPARC expression in PrSC by SPARC siRNA transfection. Expression of SPARC mRNA was detected by RT-PCR (Top). After transfection of SPARC siRNA (4×10^5 cells), SPARC concentrations in the medium were measured by ELISA (Bottom). **D**: LNCaP, DUI45, and PC-3 were cocultured with 20 nmol/L SPARC siRNA transfected PrSC for 48 hr in a transwell chamber. The cell lysates with an equal amount of proteins in cells were subjected to western blot with anti-phospho-AKTand anti-AKTantibody.

SPARC is resulted from apoptosis, PCa cells were treated with 1.0 μg/ml SPARC for 2 days, and then stained with DAPI to confirm apoptosis. However, staining of nuclei by DAPI did not detect chromatin condensation that shows apoptosis although total number of cells nuclei was decreased by 1.0 μg/ml SPARC substantially compared with vehicle (data not shown). We also assessed the expression of apoptosis-related protein, PARP, and tried to detect apoptosis in PCa cells by apoptosis assay, measures the levels of soluble caspase-cleaved fragments, however, similarly there were no apoptotic change by exogenous SPARC (data not shown). Since we confirmed that total number of cells was decreased by exogenous SPARC with DAPI stain, we analyzed the effects of exogenous SPRAC on tumor growth using

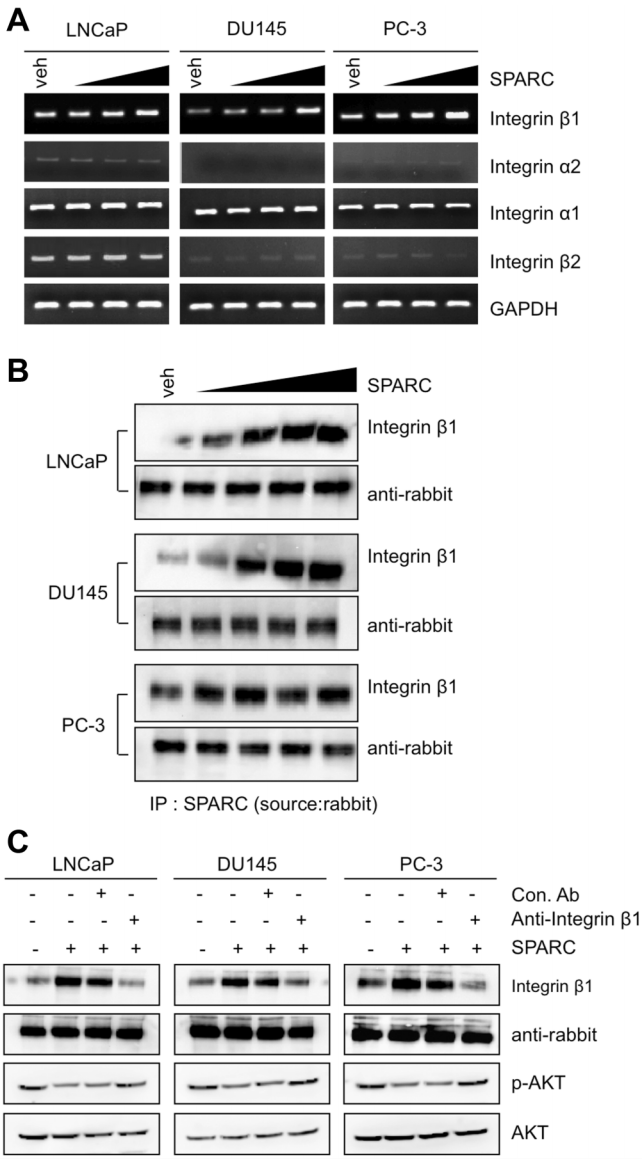

Fig. 4. Interaction of SPARC with integrin β1. **A**: After LNCaP, DU145, and PC-3 cells (4×10^5) were treated with vehicle or 0.5, 1.0, and 2.0 μg/ml SPARC for 2 days, the expression levels of integrin α1, α2, β1, and β2 mRNA were measured by RT-PCR. **B**: After cells were treated with 0, 0.5, 1.0, 1.5, and 2.0 μg/ml SPARC for 2 days, whole cell lysate from each sample were subjected to immunoprecipitation with anti-SPARC (source: rabbit) and anti-integrin β1. Total protein used for immunoprecipitation was confirmed with an anti-rabbit antibody as precipitated anti-SPARC antibody source was rabbit. **C**: Cells were treated with 1.0 μg/ml SPARC for 2 days in presence or absence of an isotype control or integrin β1–blocking antibody (10 μg/ml). Immunoprecipitation was conducted with anti-SPARC and the precipitated proteins revealed with and anti-integrin β antibodies. Whole cell lysate from each sample was also subjected to western blot analysis with anti-phospho-AKT and anti-AKTantibody.

proliferation assay kit (Fig. 5A). The proliferation rates of three kinds of PCa cells treated with 1 μg/ml SPARC were decreased compared with vehicle. To determine whether the roles of exogenous SPARC in PCa cell proliferation depends on interaction with integrin β1, cells were cultured for 12 and 72 hr with or without integrin β1-blocking antibody, nonspecific isotype control antibody and 1.0 μg/ml SPARC and then assessed to proliferation assay (Fig. 5B). The inhibited proliferation of PCa cells by treatment with SPARC was recovered by addition of integrin β1-blocking antibody, suggesting that anti-proliferative effect of exogenous SPARC is mediated through integrin β1.

Exogenous SPARC Decreases Prostate Cancer Cells Migration

To determine the effect of exogenous SPARC on migration in LNCaP, DU145, and PC-3 cells were assessed using the wound healing assay (Fig. 6A). One microgram per milliliter SPARC caused inhibition of cell migration in all three kinds of PCa cells. To further confirm this result, we performed the cell migration assay in the presence of the exogenous SPARC using a transwel chamber. As shown in Figure 6B, cell migration was also significantly decreased in the presence of 1 μg/ml SPARC. We also analyzed whether interaction between exogenous SPARC and integrin β1 affects migration in PCa cells. After cells treated with 1.0 μg/ml SPARC were cultured with integrin β1-blocking antibody or non-specific isotype control antibody for 48 hr, migration of PCa cells were assessed. As shown in Figure 6C, migration inhibited by treatment with SPARC in all PCa cells was recovered by addition of integrin β1-blocking antibody. These results suggested that exogenous SPARC repressed migration of PCa cells mediated through integrin β1.

DISCUSSION

Several studies have investigated that local changes in the physical properties of the matrix-associated factors from stromal cells might potentially lead to cancer cell progression [28,29]. Exogenous SPARC, one of the matrix-associated factors, has been reported to reduce the cell growth in ovarian cancer, neuroblastoma, and colorectal cancer [8,30,31].

Our approach was to confirm how extracelluar matrix SPARC affects PCa progression. It is known that SPARC plays important roles in development and the control of proliferation and migration with complex biological effects that are cell and tumor type specific [10]. In PCa, although Jacob et al. reported SPARC as a predominantly protumorigenic protein [21], several

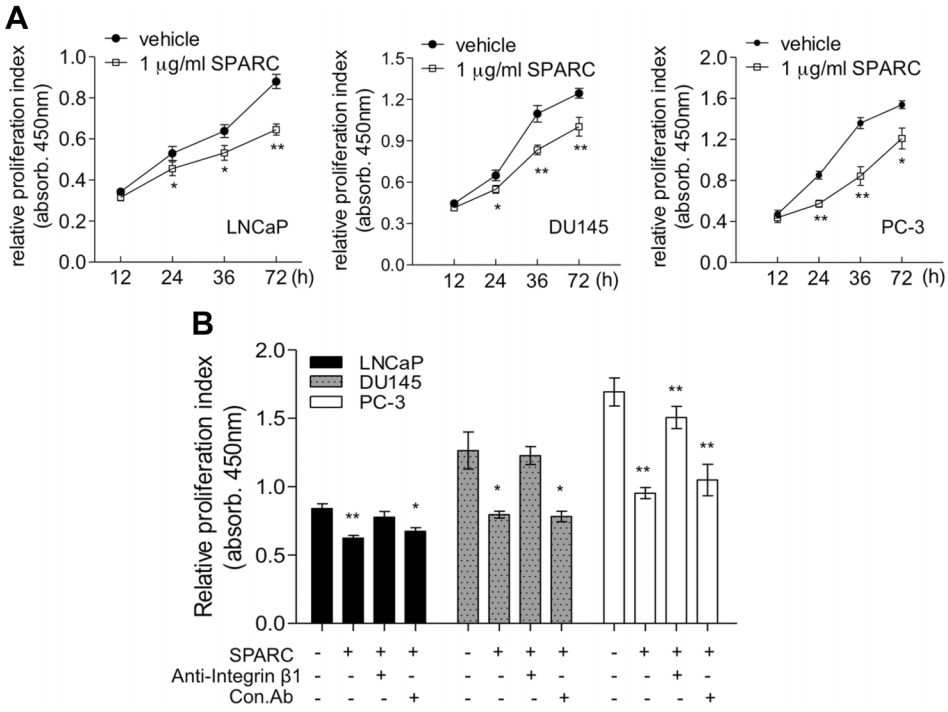

Fig. 5. Effect of exogenous SPARC on proliferation of prostate cancer cells. **A**: LNCaP, DUI45, and PC-3 cells (3×10^4/well) were cultured with vehicle (media) or 1 μg/ml SPARC in 5% FBS-containing medium for 12, 24, 36, and 72 hr. Cell proliferation was determined with a colorimetric WST-1/ECS assay. Experiments were conducted with three experimental replicates. **B**: Cells (3×10^4/well) were treated with 1.0 μg/ml SPARC for 72 hr in presence or absence of an isotype control or integrin β1–blocking antibody (10 μg/ml).

groups reported that SPARC down-regulated the proliferation and invasion of PCa cells [22,23,32], suggesting that SPARC can become the useful immunohistochemical biomarker of PCa. Of interest, normal prostate-derived stromal cells expressed high level of SPARC compared with PCa cells. Furthermore, the amount of secreted exogenous SPARC in normal prostate derived stromal cells (PrSC) was also 1.8–2.4 times higher than PCa-derived stromal cells (PCaSC-5, -6, -7, -8). Moreover, we demonstrated that exogenous SPARC suppresses cell proliferation and migration of three kinds of PCa cells (LNCaP, DU145, and PC-3). Our data support the concept that gradually diminished exogenous SPARC from PCaSC may affect PCa progression. It still remains unclear why the level of SPARC secretion from PCaSC is diminished compared with normal PrSC. Some factor from PCa cells may influence stromal cells to diminish a secretion of SPARC as well as stromal cells influencing PCa cells (Fig. 7).

Our data demonstrated that high expression of SPARC in stromal cells was correlated with suppression of AKT phosphorylation. PI3K/AKT signaling is critical to PCa cell survival and proliferation. Activated AKT translocates to the cytoplasm and nucleus and activates downstream targets involved in survival, proliferation, cell cycle progression, growth, migration [33,34]. To our knowledge, this is the first report that exogenous SPARC from stromal cells down-regulates AKT phosphorylation in PCa cells. Several previous reports associated with glioma and neuroblastoma showed that SPARC inhibits cancer cell proliferation through PTEN (phosphatase and tensin homolog deleted on chromosome 10) and AKT mediated signaling pathway. Thomas et al. reported that SPARC overexpression in glioma increased the tumor-suppressing potential of PTEN both in vitro and in vivo [35,36]. We further need to reveal whether exogenous SPARC-induced AKT dephosphorylation also regulate PTEN-related signaling pathway in PCa cells.

In the present study, we did not detect significant differences in mRNA expression of integrin α1, α2, and β2 in PCa cells by exogenous SPARC. However, integrin β1 mRNA expression was increased by exogenous SPARC. Although a number of studies have investigated the roles of integrin β1, both up-regulation and down-regulation of integrin β1 expression were reported during progression of prostate cancer [37,38]. However, dramatically reduced integrin β1 expression was observed in PCa cells [39,40] and Moran-Jones et al. [41] has shown that integrin β1

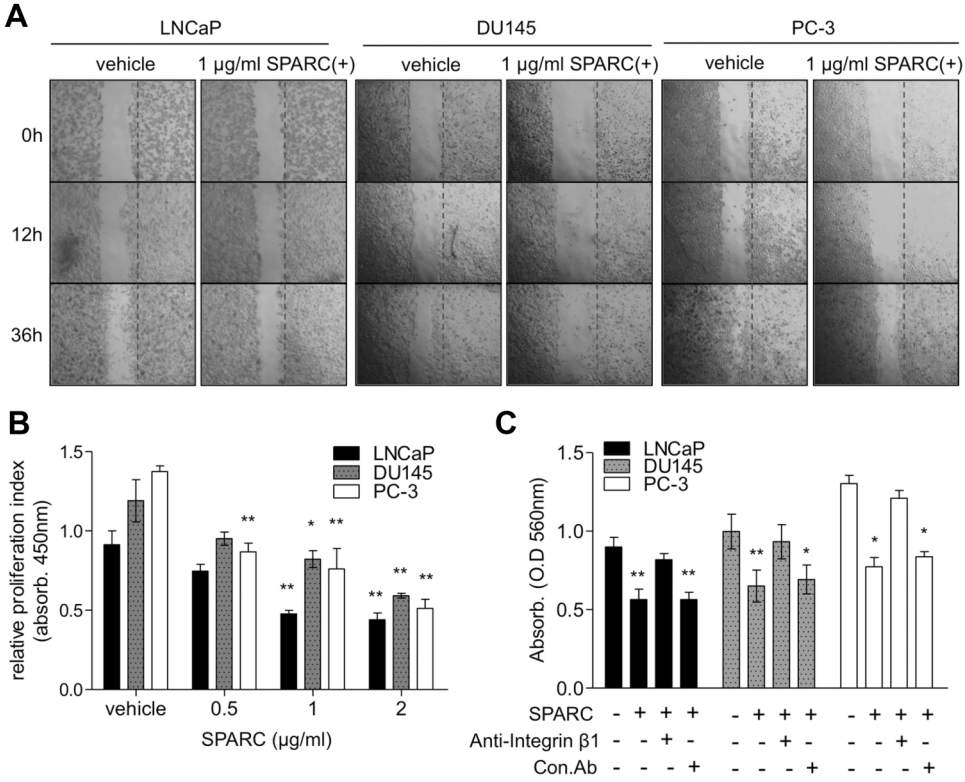

Fig. 6. Effect of exogenous SPARC on migration of prostate cancer cells. **A**: Representative photographs of migrated cells that received either control treatment or exogenous SPARC. Cells were wounded and then treated with vehicle (media) or 1 μg/ml SPARC in 5% FBS-containing medium. Images are taken immediately after scratching the cultures (0 hr), 12, and 36 hr later (original magnification, ×40). **B**: LNCaP, DU145, and PC-3 cells were cultured under vehicle or 0.5, 1.0, and 2.0 μg/ml SPARC conditions for 2 days. The cells were incubated on the 8 μm pore polycarbonate membrane in a transwell. **C**: Cells were treated with 1.0 μg/ml SPARC for 2 days in presence or absence of an isotype control or integrin β1-blocking antibody (10 μg/ml). Cell migration was assessed by transwell migration assay.

regulated normal prostate development and its loss was associated with increased rates of prostate tumor progression [25]. Our results presented here are coincident with these ideas. Integrin β1 by exogenous SPARC further adds to our understanding how integrin β1 can repress tumor progression. Moreover, our data provide the first indication of the interaction between exogenous SPARC and integrin β1 in PCa. We also confirmed that blocking the direct interaction of SPARC-integrin β1 by integrin β1-neutralizing antibody prevented SPARC from inhibiting proliferation and migration of PCa cells. These findings indicated the possibility that integrin β1 played an important role as a receptor through direct interaction with SPARC. SPARC-integrin complex has studied in some types of cancers, and SPARC-integrin β1 interaction was detected in lens epithelial cells (LEC) and astrocytes [42,43]. As a result of ours, integrin β1 could be an important bridge signaling between exogenous SPARC and PCa cells that regulates proliferation and migration of PCa.

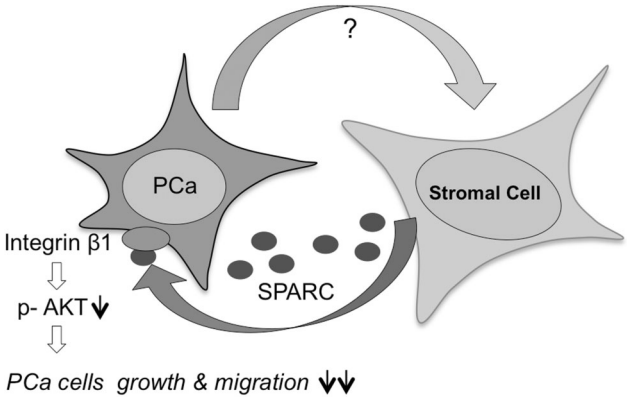

Fig. 7. Function of SPARC on prostate cancer. Mechanism of how exogenous SPARC from stromal cells effect on PCa cells growth and migration.

Our study suggests that exogenous SPARC interacted with integrin β1 in PCa cells and induced suppression of AKT phosphorylation. Moreover, the interaction suppressed proliferation and migration of PCa cells (Fig. 7). Since normal prostate-derived stromal cells secrets high level of SPARC compared with

PCa-derived stromal cells, loss of exogenous SPARC may has been linked to enhanced proliferation and migration of PCa cells. Therefore, exogenous SPARC and integrin β1 provides potential therapeutic strategies to suppress PCa progression by maintaining the level of exogenous SPARC and/or by controlling the expression, availability and affinity of integrin β1 as a receptor of exogenous SPARC. Understanding the mechanism of SPARC from microenvironment and integrin β1 in progression of PCa cells may be useful as diagnostic approaches and therapeutic indicators.

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