Secreted protein acidic and rich in cysteine (SPARC) induces epithelial-mesenchymal transition, enhancing migration and invasion, and is associated with high Gleason score in prostate cancer

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INTRODUCTION
Prostate cancer (PCa) is the fifth leading cause of death by cancer in men worldwide. Life expectancy is directly related to the appearance of metastasis in this disease. Metastasis is a process in which tumor cells detach from the primary tumor, escape to the circulation and invade distant organs to form secondary tumors. Despite advances in diagnosis and therapies, poor clinical prognosis remains for patients with metastasis. When the disease is confined to the prostate, 5-year survival is 99%; however, when metastasis arises, the 5-year survival falls to 28%. One factor that promotes metastatic progression of PCa cells is secreted protein acidic and rich in cysteine (SPARC). SPARC, also known as basement membrane-40 (BM-40) and osteonectin, is a matricellular glycoprotein that promotes collagen deposition in the stroma. Consequently, SPARC is highly expressed in tissues with high turnover of the extracellular matrix, such as bone tissue, healing wounds, and malign tumors. In addition, SPARC regulates numerous biological processes important for tumor progression, such as cell proliferation, differentiation, adhesion, migration, and angiogenesis. In tumors, SPARC can be secreted from cancer cells, the surrounding stromal cells, or both. High expression of SPARC is related to tumor progression in melanoma, pancreatic cancer, breast cancer, glioma, and others. In PCa, evidence shows SPARC promoting both pro- and anti-tumor properties. Exogenous SPARC, present in bone extracts or purified, promotes tumor progression by acting as chemotactant that directs tumor cell homing to the bone. However, exogenous SPARC also inhibits tumor progression by suppressing migration and tumor cell growth. More recently, it has been found that SPARC upregulates canonical transcription factors that induce epithelial-mesenchymal transition (EMT), such as Snail family transcriptional repressor 1 (Snail) and Snail family transcriptional repressor 2 (Slug), in melanoma and non-small cell lung cancer. EMT is a transdifferentiation program crucial for metastasis because cells undergoing EMT change their polarity, lose cell-cell contact, migrate, and invade the tumor stroma. In cancer cells, EMT activation can be induced and maintained by secreted proteins, such as cytokines and growth factors present in the tumor microenvironment, through paracrine or autocrine mechanisms. Because SPARC upregulates canonical EMT transcription factors (EMT-TFs), we propose that endogenous SPARC induces molecular and functional changes associated with EMT, in early stages of tumor development, thus promoting metastatic progression. In this study, we show that SPARC is expressed in PCa primary tumors with higher Gleason scores (Gs). Through silencing and overexpression of SPARC in PCa, we demonstrate that SPARC induces the EMT program in PCa, increasing their motility and invasive capacities in an autocrine manner.

MATERIALS AND METHODS
Tissue micro arrays (TMAs)
Formalin-fixed and paraffin-embedded samples from patients
diagnosed with PCa were obtained after radical prostatectomy surgery at the Clinical Hospital of the University of Chile, Santiago, Chile. All samples were evaluated by an expert pathologist and classified according to their GS: samples with a score of 6 were classified as low GS, samples with a score of 7 were classified as intermediate GS, and samples scored from 8 to 10 were classified as high GS. From the tissue specimens collected, we constructed two TMAs that contained 1-mm diameter cores of samples, including 23 samples of low GS, 59 samples of intermediate GS, and 26 samples of high GS, plus 12 samples of benign prostatic hyperplasia (BPH). This study was approved by the Bioethics Committees of the Faculty of Medicine and the Clinical Hospital of the University of Chile, and all patients provided informed consent.

**Immunohistochemistry (IHC)**

Tissue sections obtained from the TMAs were processed and stained in an automated IHC stainer (Benchmark GX, Ventana, Tucson, AZ, USA), according to standard procedures. Briefly, samples were dewaxed, rehydrated, and incubated for 30 min at 95°C in antigen retrieval buffer (citrate buffer, pH 8.0). After blocking, sections were probed with a specific antibody against SPARC (1:100, 335500, Life Technologies, Carlsbad, CA, USA) and nuclei were stained with hematoxylin (Sctyek laboratories, Logan, UT, USA). Digital images were obtained using a Leica DM2500 microscope (Leica, Wetzlar, Germany) and 3,3'-diaminobenzidine (DAB) signal was quantified using the software ImageJ 1.51w (NIH, Bethesda, MD, USA), with the IHC toolbox plugin.

**Cell culture**

All cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). LNCaP clone FG C (CRL1740) and 22Rv1 (CRL2505) cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 media (GIBCO, Life Technologies). DU145 (HTB81) and PC3 (CRL1435) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) F12 media (GIBCO). Both culture media were supplemented with 10% fetal bovine serum (FBS; Mediatech, Manassas, VA, USA), streptomycin-penicillin and culture media were supplemented with 10% fetal bovine serum albumin (BSA) in Tris-buffered saline (TBS) with 0.2% Tween and incubated overnight with primary antibodies diluted in blocking serum.”

**Lentiviral transduction**

Knockdown cells for SPARC were obtained through transduction with lentiviral vectors containing short hairpin RNA (shRNA) against SPARC (pLenti-U6-shRNA [h SPARC]-Rsv[GFP-Puro]), or shRNA scramble as control (pLenti-U6-shRNA [Neg-control]-Rsv[GFP-Puro]). To overexpress SPARC, cells were transduced with lentivirus containing the SPARC sequence coupled to a HA tag (pLenti-suCMV[h SPARC-HA]-Rsv[GFP-Puro]) or the empty vector as control (pLenti-suCMV[Null-control]-Rsv[GFP-Puro]). All lentiviruses were purchased from Gen Target Inc. (San Diego, CA, USA) and cells were infected using a standard procedure. Briefly, 7.5 × 10⁵ cells per well were seeded in 6-well plates. After 24 h, cells were incubated with lentiviral particles at a multiplicity of infection of three, plus 5 μg ml⁻¹ polybrene (Sigma-Aldrich, St. Louis, MO, USA) in 1 ml of culture media for 24 h. Later, cells integrating the vectors were selected using 2 μg ml⁻¹ puromycin (Sigma-Aldrich) for 24 h.

**Western blot**

Whole-cell protein was extracted using radioimmunoprecipitation assay (RIPA) buffer with Complete M mini, ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Next, 50 μg protein was loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Blots were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) with 0.2% Tween and incubated overnight with primary antibodies diluted in blocking buffer. After washing, bound primary antibodies were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and revealed with an enhanced chemiluminescence detection kit for HRP (EZ-ECL, Biological Industries, Cromwell, CT, USA). The antibodies used in this work were: anti-SPARC (1:500, sc-25574, Santa Cruz, Dallas, TX, USA), anti-hemagglutinin (HA; 1:1000, H6908, Sigma-Aldrich), anti-zinc finger E-box binding homeobox 1 (Zeb1; 1:1000, 14974182, eBioscience, Thermo Fisher, Waltham, MA, USA), anti-Snail (1:500, sc-393127, Santa Cruz), anti-Slug (1:500, sc-166479, Santa Cruz), anti-E-cadherin (1:1000, 610181, BD Transduction Laboratories, San Jose, CA, USA), anti-N-cadherin (1:1000, 333900, Life Technologies), anti-Vimentin (1:2000, ab8978, AbCam, Cambridge, UK), anti-Actin (1:5000, MP Biomedicals, Santa Ana, CA, USA), anti-mouse HRP (1:10000, Jackson ImmunoResearch, West Grove, PA, USA), and anti-rabbit HRP (1:10000, Jackson ImmunoResearch).

**RNA extraction and quantitative real-time reverse transcriptase PCR (RT-qPCR)**

Total RNA was extracted from cells using TRizol (Ambion, Life Technologies). Three thousand nanograms of cDNA was synthesized using the kit cDNA Affinity Script QPCR (Agilent Technologies, Santa Clara, CA, USA) and 100 ng of cDNA was amplified by qPCR using the kit Brilliant II SYBR Green qPCR Master Mix (Agilent Technologies). The housekeeping gene pumilio RNA binding family member 1 (PUM1) was used as a normalizer and the results were analyzed using the ΔΔCt method. The primer sets used for the qPCRs are detailed in Table 1.

**Table 1: The primer sets used for the quantitative polymerase chain reactions**

| Gene name (protein common name) | Abbreviation | Forward primer | Reverse primer |
|---------------------------------|--------------|----------------|----------------|
| Secreted protein acidic and rich in cysteine (SPARC)  | SPARC | 5'-AAG CGA AGA AGA GGT GGT GGT-3' | 5'-GCA AAG TGG CAG GAA GA-3' |
| Cadherin 1 (E-cadherin) | CDH1 | 5'-GGA CAG TTC CTG AGG CAT CA-3' | 5'-ATT CGG GCT TGT TGT CAT TC-3' |
| Cadherin 2 (N-cadherin) | CDH2 | 5'-GCC AAG GCA AGT GCG G-3' | 5'-CAT TTC AGT CAG CTC GGC-3' |
| Vimentin (Vimentin) | VIM | 5'-ACA GAG TGA GGA GCC TGG AGA CCG A-3' | 5'-CAG TAT TGG AGA GTG CAG GCC TC-3' |
| Keratin 18 (Cytokeratin 18) | KRT18 | 5'-GCA AAG GCA AGT GCC TGG AGA CCG A-3' | 5'-GCA TAT TGG AGA GTG CAG GCC TC-3' |
| Zinc finger e-box binding homebox 1 (Zeb1) | ZEB1 | 5'-TCC ACA GTG GAG AGC AGA CA-3' | 5'-GCG TGG TGA TGC TGA AAG AG-3' |
| Snail family transcriptional repressor 1 (Snail) | SNAI1 | 5'-TCC CAG CAC CCC TAC GAC CAG-3' | 5'-GCC TTT CCC ACT GTC CTC ATC-3' |
| Snail family transcriptional repressor 2 (Slug) | SNAI2 | 5'-CTC CAT TCC AGC CCC AGC TAC-3' | 5'-AGC CAC TGT GGT CTT TGG AG-3' |
| Matrix metalloproteinase 2 (Matrix metalloproteinase-2) | MMP-2 | 5'-AAG CCG AAG TGG GAC AAG AA-3' | 5'-ACT TGG AAG GCA CGA GCA AA-3' |
| Matrix metalloproteinase 7 (Matrix metalloproteinase-7) | MMP-7 | 5'-TGG GAC ATT CCT CTG ATC CT-3' | 5'-TGA ATG GAT GTT CCT GCT GA-3' |
| Pumilio RNA binding family member 1 (Pumilio homolog) | PUM1 | 5'-CAG TCG TCC TGA GGA GAA AA-3' | 5'-CTG ACG TGG GGC GTG AGT AA-3' |
**Indirect immunofluorescence and fluorescent staining**

Cells were seeded in 12-mm coverslips at a confluence of 50%. After 24 h, cells were fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, washed, and blocked with 3% BSA in PBS for 30 min. Cells were incubated overnight with primary antibodies against SPARC (1:100, sc-25574, Santa Cruz), Zeb1 (1:50, sc-25388, Santa Cruz) or Ki67 (1:50, sc-15402, Santa Cruz), washed and incubated for 45 min with secondary antibody Alexa Fluor 594 (1:500, A21207, Life Technologies). 4',6-diamidino-2-phenylindole (DAPI; 1:10000, sc3598, Santa Cruz) and Phalloidin (50 μg ml⁻¹, P-1951, Sigma–Aldrich) were used for nuclear and cytoplasmatic staining, respectively. Cell area and circularity were quantified using the software ImageJ 1.51w.

**Wound healing assay**

Cells were seeded in 24-well plates and cultured on confluence. A scratch was made with a pipette tip and the wound was photographed every 12 h for 3 days. Wound area was quantified using the software ImageJ 1.51w.

**Transwell migration assay**

For transwell migration assay, 5 × 10⁴ cells per well were seeded in the upper chamber of a 96-well CytoSelect™ (Cell Biolabs, San Diego, CA, USA) plate with 8-μm pore membranes. Cells in the upper chamber were kept in culture media without FBS, whereas in the lower chamber, culture media with 10% FBS was placed as chemoattractant. After 24 h, transmigrated cells were resuspended and dyed with CyQuant® GR Dye (Cell Biolabs). Fluorescence at 485/528 nm was quantified in a BioTek Synergy HT plate reader (BioTek, Winooski, VT, USA).

**In vitro invasion assay**

Invasion assay was performed in a 96-well CytoSelect™ (Cell Biolabs) plate with 8-μm pore membranes coated with the basement membrane, following the same protocol used for the transwell migration assay described above.

**MTT viability assay**

Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). For this, 1 × 10⁴ cells per well were seeded in a 48-well plate. After 24 h, 48 h and 72 h, cells were washed in PBS. Then, 100 μl of MTT working solution (15 μl MTT [5 mg ml⁻¹] in 500 μl Locke solution) was added and cells were incubated for 3 h at 37°C. Afterward, the solution was discarded, formazan crystals were resuspended in 100 μl dimethyl sulfoxide and the absorbance at 550 nm was measured in a BioTek Synergy HT plate reader (BioTek).

**Trypan blue exclusion test**

For the trypan blue exclusion test, 5 × 10⁴ cells per well were seeded in 12-well plates and cell growth was monitored every 24 h for 3 days, by counting the total number of viable cells per well. For this, cells were detached with trypsin and resuspended in 1 ml culture media. Next, 10 μl trypan blue (0.4%; Sigma–Aldrich) was added to 10 μl of cell suspension and 10 μl of this mix was loaded in a hemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). Only viable (nonstained) cells were considered for the count.

**Statistical analyses**

Data analysis was performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). For TMA analysis, data distribution was evaluated with the D’Agostino-Pearson normality test and differences between groups was analyzed with the Kruskal–Wallis test. In all other experiments, data are expressed as mean ± standard deviation, of at least three independent experiments. For continuous data, Mann–Whitney U test, Student’s t-test, and one-way ANOVA or two-way ANOVA were used to analyze differences between groups. In all cases, P ≤ 0.05 was considered statistically significant.

**Ethical and safety considerations**

All procedures were approved by the Ethics Committee for Research on Human Beings and the Risk Prevention and Biosafety Unit of the Faculty of Medicine of the University of Chile.

**RESULTS**

**Expression of SPARC in PCa samples is associated with high GS**

To evaluate SPARC expression in human PCa samples, two TMAs including prostate tissue specimens of 120 patients were constructed and immunohistochemistry staining of SPARC was performed (Figure 1a and 1b). PCa specimens present positive intracellular staining for SPARC (Figure 1a). Furthermore, quantification of DAB staining (Figure 1c) revealed that SPARC expression is increased in samples of intermediate and high GS compared with those of nonneoplastic prostate disease (BPH) (P ≤ 0.001, Kruskal–Wallis test) or PCa samples of low GS (P ≤ 0.01, Kruskal–Wallis test).

**Stable silencing and overexpression of SPARC change morphological features in PCa cell lines**

Because SPARC showed high expression in the more aggressive PCs, we aimed to determine the biological effects of SPARC *in vitro*, modifying its expression in PCa cell lines. For this, we first determined the base levels of SPARC in different PCa cell lines. Four frequently-used cell lines were selected: 22Rv1, LNCaP, DU145, and PC3. PC3 cells have the highest expression of SPARC mRNA and protein.
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(Figure 2a and 2b). Conversely, 22Rv1, LNCaP and DU145 cells have very low SPARC expression. Based on this, we chose the PC3 cell line to knock down SPARC through transduction with lentiviral vectors expressing a shRNA directed against SPARC. The LNCaP cell line was used to overexpress SPARC by transduction with lentiviral vectors expressing the SPARC sequence. Western blot (Figure 2c), RT-qPCR (Figure 2d) and immunofluorescence (Figure 2e) confirmed the changes in the intracellular levels of SPARC (P ≤ 0.001, one-way ANOVA). In addition, morphological features of cells with SPARC silencing and overexpression were assessed. PC3 shScramble cells have a fusiform shape, while PC3 shSPARC cells are more circular and bigger (P ≤ 0.001, Mann–Whitney U test), which is consistent with an epithelial morphology. Conversely, LNCaP cells with overexpression of SPARC are smaller (P = 0.017, Mann–Whitney U test), although the circularity does not change (P = 0.779, Mann–Whitney U test) (Figure 2f–2h).

SPARC induces EMT in PCa cell lines

To determine whether SPARC could modulate the cellular phenotype, classical EMT markers, the cell–cell adhesion molecules E-cadherin and N-cadherin and the intermediate filaments vimentin and cytokeratin 18 were evaluated in the established cell lines. Knockdown of SPARC in PC3 cells resulted in increased E-cadherin (P = 0.013, Student’s t-test) and cytokeratin 18 (P ≤ 0.01, Student’s t-test), accompanied by a decrease in N-cadherin and vimentin (P ≤ 0.001, Student’s t-test). Conversely, overexpression of SPARC in LNCaP cells downregulated E-cadherin and cytokeratin 18 (P ≤ 0.001, Student’s t-test), and upregulated vimentin (P = 0.019, Student’s t-test). Although SPARC overexpression

Figure 2: Basal expression, silencing, and overexpression of SPARC in PCa cell lines. (a) Representative image of western blot against SPARC in PCa cell lines 22Rv1, LNCaP, DU145 and PC3. (b) RT-qPCR of SPARC in PCa cell lines. ∆ΔCt was obtained after normalizing to PUM1 and PC3 cell lines (n = 3). (c and d) PC3 cells were stable transduced with shRNA against SPARC (ShSPARC) or scramble (ShScr). LNCaP cells were stable transduced with SPARC sequence (SPARC-HA) or an empty vector (Null). Parental cells (Input) were used as control. (e) Representative images of western blot against SPARC in the different cell lines produced. Quantification of optic density was normalized to β-actin and parental cells, numbers show median (n = 3). (d) SPARC mRNA expression assessed through RT-qPCR (n = 3); ***P ≤ 0.001 (one-way ANOVA). (e) Representative images of phase contrast and immunofluorescence against SPARC in transduced and parental cell lines. (f) Representative images of phase contrast and immunofluorescence against SPARC in transduced and parental cell lines. (g) Cell area and (h) circularity of transduced PC3 and LNCaP cells stained with DAPI and phalloidin. Scale bars = 20 μm in e and f. (g) Cell area and (h) circularity of transduced PC3 and LNCaP cells stained with DAPI and phalloidin (n = 50); *P = 0.017; ***P ≤ 0.001, NS: not significant, P > 0.05 (Mann–Whitney U test). SPARC: secreted protein acidic and rich in cysteine; PUM1: pumilio RNA binding family member 1; ANOVA: analysis of variance; RT-qPCR: quantitative real-time reverse transcriptase PCR; ShSPARC: shRNA against SPARC; ShScr: shRNA against scramble; DAPI: 4’,6-diamidino-2-phenylindole.
increased N-cadherin expression, it was not possible to quantify these changes because parental and null LNCaP cells do not show N-cadherin expression by RT-qPCR or western blot (Figure 3a and 3c). Together, the changes in EMT markers expression indicate that SPARC induces EMT in PCa cell lines. Because the changes in protein expression that occur during the EMT are regulated by transcriptional factors that inhibit the transcription of genes associated with epithelial phenotype and induce the transcription of mesenchymal genes, we assessed whether SPARC can modulate the expression levels of the EMT-TFs Zeb1, Snail, and Slug. SPARC knockdown in PC3 cells decreased the mRNA of the transcription factors Zeb1 (P ≤ 0.001, Student’s t-test), Snail (P = 0.026, Student’s t-test), and Slug (P ≤ 0.001, Student’s t-test) (Figure 3d). However, at protein level, only decrease of Zeb1 was observed (P ≤ 0.001, Student’s t-test) (Figure 3b). Conversely, the stable overexpression of SPARC in LNCaP cells increased the expression of Zeb1 (P ≤ 0.001, Student’s t-test), Snail (P = 0.017, Student’s t-test), and Slug (P = 0.026, Student’s t-test), both at the mRNA and protein levels (Figure 3b and 3d). As active EMT-TFs are localized in the nucleus, Zeb1 expression, through indirect immunofluorescence, was also evaluated. As expected, in normal PC3 cells and LNCaP cells with SPARC overexpression, a strong Zeb1 signal was observed into the nuclei, whereas in normal LNCaP and in PC3 cells knockdown for SPARC, only a diffuse cytoplasmic signal was observed (Figure 3e).

**SPARC does not induce changes in the proliferation of PCa cells**

Given that SPARC upregulates the expression of EMT-TFs, which can directly inhibit proliferation, the effect of SPARC on cell proliferation in PCa cell lines was evaluated. We observed that neither silencing nor overexpression of SPARC in PCa cells modifies *in vitro* proliferation, evaluated by three different methods: MTI (P = 0.993, two-way ANOVA), Trypan Blue exclusion test (P = 0.999, two-way ANOVA), and immunofluorescence against Ki67 (P = 0.842, one-way ANOVA) (Figure 4).

**SPARC increases the motility and *in vitro* invasive capacity of PCa cells**

Because high motility is one of the distinctive features of mesenchymal cells, we evaluated whether SPARC-induced mesenchymal phenotype is accompanied by an increase in cell motility. We assessed the motility capacities of PCa cells with SPARC knockdown and overexpression through a wound closure test. Silencing of SPARC in PC3 decreased the motility of these cells (P ≤ 0.001, two-way ANOVA), while the overexpression of SPARC in LNCaP cells increased it (P ≤ 0.001, two-way ANOVA) (Figure 5a and 5b). To confirm these findings, we performed a migration test with a modified Boyden chamber, using FBS as chemotactrant. As expected, cells expressing SPARC showed a high ability to transmigrate through the chamber pores in response to the chemotactrant stimulus (PC3 cells: P ≤ 0.01; LNCaP cells: P = 0.018, Student’s t-test) (Figure 5c).

To determine whether SPARC also increases the invasive capacity, a modified Boyden chamber coated in basement membrane was used. SPARC-knockdown PC3 cells showed lower ability to invade through the matrix than normal PC3 cells (P = 0.047, Student’s t-test)

**Figure 3:** Expression of EMT markers and EMT-TFs in PCa cell lines with SPARC knockdown and overexpression. (a) Western blot of EMT markers E-cadherin, N-cadherin and vimentin and (b) EMT-TFs Zeb1, Snail and Slug in PC3 cells transduced with shRNA against SPARC, LNCaP cells transduced with the SPARC sequence and its respective controls. Quantification of optic density was normalized to β-actin and parental cells, numbers show median (n = 3). Relative mRNA expression of (c) EMT markers and (d) EMT-TFs, assessed by RT-qPCR (n = 3); *P* ≤ 0.05, **P** ≤ 0.01, and ***P** ≤ 0.001 (Student’s t-test). (e) Representative images of immunofluorescence against Zeb1 in PC3 with SPARC silencing and LNCaP cells with SPARC overexpression and its respective controls. Scale bars = 20 μm. Zeb1: zinc finger E-box binding homeobox 1; EMT: epithelial-mesenchymal transition; EMT-TFs: EMT transcription factors; shRNA: short hairpin RNA; SPARC: secreted protein acidic and rich in cysteine; RT-qPCR: quantitative real-time reverse transcriptase PCR; VIM: Vimentin; ShSPARC: shRNA against SPARC; ShScr: shRNA against scramble; DAPI: 4’,6-diamidino-2-phenylindole; Snail: Snail family transcriptional repressor 1; Slug: Snail family transcriptional repressor 2.
(Figure 5d), but LNCaP cells with SPARC overexpression showed no changes in their invasive capacity ($P = 0.435$, Student’s $t$-test).

Because matrix metalloproteinases (MMPs) are crucial for tumor cell invasion, we evaluated their expression in PCa cells with SPARC silencing and overexpression. SPARC knockdown downregulated MMP-2 and MMP-7 mRNA levels in PC3 cells ($P < 0.001$, Student’s $t$-test). However, no changes were observed in the expression of MMP-2 ($P = 0.102$, Student’s $t$-test) and MMP-7 ($P = 0.084$, Student’s $t$-test) in LNCaP cells with SPARC overexpression (Figure 5e).

DISCUSSION

SPARC is a matricellular protein highly expressed in bone tissue and described as a chemoattractant factor that could promote the arrival of PCa cells in the bone marrow.$^{11,14,15}$ However, there is no consensus regarding its contribution during the early stages of tumor progression.$^7$

In this study, we found that SPARC is expressed in primary tumor biopsies from PCa patients, being high in those of intermediate and high GS. Our results confirm those of Sung et al.$^8$ who found that PCa tissue expresses more SPARC than normal prostate tissue, and of Derosa et al.$^{25}$ who showed that SPARC is more expressed by poorly-differentiated PCa compared with well-differentiated PCa tumors. Conversely, Shin et al.$^{16}$ found that normal prostates have a higher intensity of SPARC immunostaining compared with PCa tissue. Because several other studies have linked SPARC expression with both normal and tumor tissue,$^{7,13}$ it is clear that SPARC expression varies during cancer progression and might exert different effects in this disease.

In the PCa cell lines studied in this work, we observed that SPARC is highly expressed in PC3 cells compared with the other three PCa cell lines. This result is consistent with the findings of Thomas et al.$^{26}$ who found that PCa cells obtained from bone metastases such as PC3 have higher expression of SPARC compared with cells obtained from primary tumors or metastasis in other organs. However, because we found that primary tumors of intermediate and high GS also express SPARC, it is possible that this increased expression of SPARC might be acquired during this earlier stage of tumor progression and not once in the bone. To investigate the effects of tumor SPARC in PCa cells, we developed an in vitro model of silencing and overexpression of SPARC. In our model, we found that SPARC expression by PCa cells leads to morphological, molecular, and functional changes associated with EMT. Our results are in agreement with recent studies in head and neck cancer and non-small cell lung cancer in which SPARC induces phenotypic and molecular changes associated with EMT.$^{20,27}$ The present work is the first report of EMT induction by SPARC in PCs.

In this study, we observed that SPARC knockdown decreases the expression of Zeb1, Snail and Slug at the mRNA level. However, at the protein level, only Zeb1 is downregulated. Snail and Slug can...
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CONCLUSION
Our data show that SPARC is highly-expressed in intermediate and high GS and induces EMT in PCa cells. In addition, SPARC induces PCa cell migration and invasion without affecting cell proliferation in vitro. These results indicate that SPARC regulates key events during tumor progression and therefore might play an important role in the
aggressiveness of PCa. Further molecular studies of the relationship between SPARC and Zeb1 should be conducted to better understand its contribution to tumor progression.

AUTHOR CONTRIBUTIONS

FLM designed and performed all the experiments and statistical analysis, and wrote the manuscript. MJT participated in experimental work and helped to draft the manuscript. EAC participated in the design and helped to draft the manuscript. HRC conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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